16. (Twice Amended) The method of claim 14, wherein the cells are stem cells, nuclear transfer donor cells, tumor cells or transformed cells.

30. (Amended) The method of claim 1, wherein the cell is selected from the group consisting of a primary cell, an immortalized cell, an embryonic cell, a stem cell, a transformed cell and a tumor cell.

REMARKS

A check for the fee for a three month extension of time accompanies this response. Any additional fees that may be due in connection with filing this paper or with this application during its entire pendency may be charged to Deposit Account No. 50-1213. If a Petition for extension of time is required, this paper is to be considered such Petition, and any fee charged to Deposit Account No. 50-1213.

Claims 1-16 and 30 are pending in this application. Claims 1, 3, 9, 10, 16 and 30 are amended to correct minor typographical and grammatical errors. Claim 1 also is amended herein to more particularly point out the intended subject matter. Basis for the amendment of claim 1 may be found throughout the specification, which provides methods designed for delivering large nucleic acid molecules into a cell, including methods of monitoring delivery of nucleic acid and expression from the nucleic acid. Particular basis may be found, for example, on page 17 , which recites: "A variety of methods for delivering nucleic acids, particularly large nucleic acids, such as artificial chromosomes, including ACes (formerly designated SATACS) are provided" (lines 23-25).

Particular basis for "large nucleic acid molecules" also can be found, for example, at page 9, line 30 through page 10, line 16, which defines "large nucleic acids molecules as follows:

> As used herein, the term "large nucleic acid molecules" or "large nucleic acids" refers to a nucleic acid molecule of at least about 0.5 megabase pairs (Mbase) in size, greater than 0.5 Mbase, including nucleic acid molecules at least about 0.6. 0.7, 0.8, 0.9, 1, 5, 10, 30, 50 and 100, 200, 300, 500 Mbase in size. Large

> nucleic acid molecules typically may be on the order of about 10 to about 450 or more Mbase, and may be of various sizes, such as, for example, from about 250 to about 400 Mbase, about 150 to about 200 Mbase, about 90 to about 120 Mbase, about 60 to about 100 Mbase and about 15 to 50 Mbase.
>
> Examples of large nucleic acid molecules include, but are not limited to, natural chromosomes and fragments thereof, especially mammalian chromosomes and fragments thereof which retain a centromere and telomeres, artificial chromosome expression systems (ACes; also called satellite DNA-based artificial chromosomes (SATACs); see U.S. Patent Nos. 6,025,155 and 6,077,697), mammalian artificial chromosomes (MACs), plant artificial chromosomes, insect artificial chromosomes, avian artificial chromosomes and minichromosomes (see, *e.g.*, 5,712,134, 5,891,691 and 5,288,625).

Claim 9 is amended to more particularly point out the claimed subject matter. Claim 9 is amended to include the phrase "introducing the labelled large nucleic acid molecules" to provide reflect the antecedent basis provided in Claims 1 and 6, from which Claim 9 depends. Basis for "introducing labelled large nucleic acid molecules" with a cationic delivery agent as recited in Claim 9 also is found in the specification, *e.g.*, at page 20, line 4 through page 22, line 4 and also in the working examples, Table 1, pages 54-55.

The amendment of the specification corrects minor typographical errors. No new matter is added.

An executed DECLARATION under 37 C.F.R. §1.132 of Vanderbyl, with reference publication, is attached hereto, as is a marked up copy per 37 C.F.R. §1.121 of the amended paragraph and claims. A Supplemental Information Disclosure Statement also is filed on the same day herewith under separate cover.

OBJECTION TO CLAIMS 1, 3 AND 30

Claims 1, 3 and 30 are objected to because of informalities within the claims. This objection has been rendered moot by amending the claims to correct the minor typographical errors pointed to by the Examiner.

PROVISIONAL REJECTION OF CLAIMS 1-8, 11-16 AND 30 UNDER STATUTORY TYPE (35 U.S.C. § 101) DOUBLE PATENTING

Claims 1-8, 11-16 and 30 are provisionally rejected under statutory type (35 U.S.C. § 101) double patenting as allegedly being unpatentable over claims 1-8, 11-16 and 30 of copending Application No. 10/086,745. Specifically, it is alleged that claims 1-8, 11-16 and 30 of copending Application No. 10/086,745 are identical in scope to the claims of the instant application.

In the Election and Preliminary Amendment filed responsive to the Requirement for Restriction that issued September 19, 2002, in connection with the above-captioned application, Applicant stated that Claims 17-29, 31 and 32, which were cancelled as being drawn to non-elected subject matter, would be prosecuted in the aforementioned co-pending U.S. application Serial No. 10/086,745. Upon receipt of an Action in co-pending U.S. application Serial No. 10/086,745 on the merits, claims of identical scope to any pending in this case will be cancelled, thereby obviating any statutory double patenting issues, if any, between the pending claims of the two applications.

THE REJECTION OF CLAIMS 1-16 and 30 UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 1-16 and 30 are rejected under 35 U.S.C. § 112, first paragraph because it is alleged that the specification does not describe the subject matter in such a way as to enable one skilled in the art to obtain gene expression from a labelled nucleic acid molecule introduced into a cell. To support this allegation, the Office Action cites Felgner *et al.* (WO 99/13719), which allegedly demonstrates that standard methods of labeling DNA do not permit detection of structurally and functionally intact plasmid. Additionally, the Office Action cites Neves *et al.* (*Bioconjugate Chem* (2000) *11:51-55*), which allegedly demonstrates that labeling DNA with either tetraflurobenzylamido-lissamine or rhodamine-labelled nucleotides reduced or abolished reporter gene expression, respectively, and furthermore, cites Zelphati *et al.* (*Hum. Gene*

Therap (1999) *10:15-24*), which allegedly provides that labeling methods reduce or destroy the ability of DNA to be transcribed.

It is further alleged that while the working examples provide a method of introducing labelled ACes carrying a reporter gene and Applicant states that reporter gene expression was measured, no evidence of detection of reporter gene expression from labelled ACes is provided. The Examiner urges that the statement by Applicant at page 35 of the specification, indicating that labelling of ACes does not affect gene expression, is not supported by data. The Examiner concludes that the lack of supporting data in the specification, when combined with the teachings in the art indicating that incorporation of labeled nucleotide analogs adversely affects gene expression, shows that the degree of experimentation by the skilled artisan to achieve expression from labelled nucleic acid molecules is undue.

This rejection is respectfully traversed. Although not needed, a DECLARATION under 37 C.F.R. §1.132 of Vanderbyl demonstrating such results is provided.

Relevant law

To satisfy the enablement requirement of 35 U.S.C § 112, first paragraph, the specification must teach one of skill in the art to make and use the invention without undue experimentation. Atlas Powder Co. v. E.I. DuPont de Nemours, 750 F.2d 1569, 224 USPQ 409 (1984). This requirement can be met by providing sufficient disclosure, either through illustrative examples or terminology, to teach one of skill in the art how to make and how to use the claimed subject matter without undue experimentation. This clause does not require "a specific example of everything *within the scope* of a broad claim." In re Anderson, 176 USPQ 331, at 333 (CCPA 1973), emphasis in original. Rather, the requirements of § 112, first paragraph "can be fulfilled by the use of illustrative examples **or** by broad terminology." In re Marzocchi et al., 469 USPQ 367 (CCPA 1971)(emphasis added).

Further, because "it is manifestly impracticable for an applicant who discloses a generic invention to give an example of every species falling within it, or even to name every such species, it is sufficient if the disclosure teaches those skilled in the art what the invention is and how to practice it." In re Grimme, Keil and Schmitz, 124 USPQ 449, 502 (CCPA 1960). Thus, there is no doubt that a patentee's invention may be broader than the particular embodiment shown in the specification. A patentee not only is entitled to narrow claims particularly directed to the preferred embodiment, but also to broad claims that define the invention without a reference to specific instrumentalities. Smith v. Snow, 294 U.S. 1, 11, 24 USPQ 26, 30 (1935).

Thus, there is no requirement for disclosure of every species within a genus. Applicant is entitled to claims are commensurate in scope not only with what applicant has specifically exemplified, but commensurate in scope with that which one of skill in the art could obtain by virtue of that which the applicant has disclosed.

Furthermore, statements and disclosure in an application are presumed true:

> [a]s a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and describing the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.
>
> . . . it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure (In re Marzocchi & Horton, 58 CCPA 1069, 439 F. 2d 220, 169 USPQ 367, 369-370 (1971)).

The inquiry with respect to scope of enablement under 35 U.S.C. §112, first paragraph, is whether it would require undue experimentation to make and use the claimed invention. A considerable amount of experimentation is permissible, particularly if it is routine experimentation. The amount of experimentation that is permissible depends upon a number of factors, which include: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability of the art, and the breadth of the claims. Ex parte Forman, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986); see also In re Wands, 8 USPQ2d 1400 (Fed. Cir. 1988).

Analysis

1. Application of the Above-noted Factors

Scope of the claims

Claims 1-16 and 30 are directed to methods for detecting or determining nucleic acid delivery and expression in a cell by introducing labelled large nucleic acid molecules, detecting labelled cells and measuring reporter gene product to measure the delivery and expression, respectively. The dependent claims further specify labeling the DNA using iododeoxyuridine or bromodeoxyuridine and the use of flow cytometry, fluorometry, cell imaging or fluorescence spectroscopy for detection of labelled DNA. The dependent claims also specify types of reporter genes including fluorescent reporter genes, such as green, red and blue fluorescent proteins, and enzymes such as β-galactosidase, luciferase and alkaline phosphatase. The dependent claims further specify types of delivery agents and specific cell types to which the DNAs are delivered. Additionally, the dependent claims specify that the delivered nucleic acid may be natural chromosomes, artificial chromosomes, chromosome fragments, or naked DNA greater than about 0.6 megabases in size.

Level of Skill

The level of skill in this art is recognized to be high (see, *e.g.*, Ex parte Forman, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986)). The numerous articles and patents made of record in this application address a highly skilled audience and further evidence the high level of skill in this art.

Teachings of the specification

The specification describes the delivery of DNA, including labelled DNA into cells and the expression of gene products encoded therein. The teachings of the specification describe how to: introduce labelled large nucleic acid to a cell; measure the delivery of the nucleic acid to the cell by detecting labelled cells; and determine the expression of the product of the reporter gene encoded by the nucleic acid.

Each of these steps are described in detail in the specification. The steps of the methods claimed herein are described in the specification in detail and the specification further provides working examples of particular embodiments thereof, including the steps of: introducing labelled nucleic acid into a cell; detecting the labelled nucleic acid as an indication of delivery; and measuring reporter gene product as an indication of gene expression. The specification also describes methods of detecting labelled nucleic acids, the use of various reporter genes, the use of different cell types, the use of different delivery agents, including various cationic lipids and the use of large nucleic acid molecules such as artificial chromosomes.

In addition, the specification exemplifies the steps of the method in numerous working examples. For example, the specification discloses methods for labeling large nucleic acid molecules with iododeoxyuridine and bromodeoxyuridine; methods of delivering labelled large nucleic acids to a variety of cell types using delivery agents such as cationic lipids, ultrasound and electroporation; methods of detecting nucleic acid delivery into the cell including

flow cytometry; and methods of detecting gene expression of reporter genes such as GFP and β-galactosidase encoded by the labelled DNA.

A) Methods for Labeling Nucleic Acids

The specification teaches methods for preparing labelled nucleic acids. Labeling includes the use of directly detectable labels, such as labeling with radioactivity (page 33, line 10) , or labels that are detectable upon binding to a cognate receptor (*e.g.*, antigen-antibody complex formation; page 33, lines 16-20). To illustrate the methods, the specification describes in extensive detail the the preparation of large nucleic acid molecules and the generation of cell lines with large nucleic acid molecules such as ACes (Example 1, section B, pages 40-41), labeling of DNA by the incorporation of iododeoxyuridine and bromodeoxyuridine (Example 7, page 52) and the purification of ACes (Example 1, section C, pages 43-45) and labelled ACes (Example 7, pages 52-53).

The specification further teaches methods for the delivery of labelled nucleic acids. These methods include the use of delivery agents such as cationic compounds (page 19, line 24 through page 22, line 4), the use of ultrasound (page 22, line 9 through page 23, line 19) and the use of electroporation (page 23, lines 20-26). The specification describes *in vitro*, *ex vivo* and *in vivo* delivery methods using these agents (page 23, line 27 through page 28, line 9). Additionally, these methods are exemplified in the Examples. Example 4 provides detailed procedures for transfection (page 46, line 5 through page 47, line 20) and transfection of labelled DNA is exemplified (page 47, lines 21-25, using the described methods). Furthermore, in Example 7, artificial chromosomes are labelled with either iododeoxyuridine (IdU) or bromodeoxyuridine (BrdU) (page 52, line 10 through page 53, line 14) and then transfected by a variety of methods (page 53, line 15 through page 55, line 5).

B) Methods for Measuring Labelled Nucleic Acid Delivery

The specification teaches methods for measuring the delivery of nucleic acid into cells by detecting labelled cells. The methods include using

microscopy and flow cytometry for detection of labelled cells (page 28, line 10 through page 29, line 12). Various parameters such as transfer efficiency, clonogenicity, viable fraction, potential transfection efficiency and chromos index for assessing the efficiency of delivery also are provided in detail (page 29, line 13 through page 33, line 2). Additionally, these methods are exemplified in Example 4 where cells transfected with IdU labelled ACes are assessed for the percentage of transfected cells using flow cytometry (page 47, line 21 through page 48, line 4). Additionally, Example 7 describes the measurement of DNA delivery (page 55, line 7 through page 56, line 5) and then details the results of such measurements in Table 2 of Example 7 (pages 56-57).

C. Methods for Measuring Gene Expression from Labelled or Non-Labelled Nucleic Acids Delivered to Cells

The specification teaches that reporter gene expression can be measured to assess expression of gene(s) encoded by the labelled large nucleic acid molecules that are delivered to cells. The specification describes numerous reporters that may be used including GFP, β-galactosidase, CAT and others (page 34, lines 21-28). The specification further teaches that reporter gene expression may be measured from labelled nucleic acid molecules (page 34, line 29 through page 35, line 15) and that labeling the nucleic acid molecules does not interfere with reporter gene expression (page 35, lines 9-15). Additionally, measurement of reporter genes is exemplified in several of the examples, including Example 1 (measurement of GFP; page 41, line 21 through page 42, line 23), Example 4 (measurement of β-galactosidase; page 47, lines 18-20), Examples 5 and 6 (GFP measurements; page 49, lines 26-31 and page 51, lines 6-8, respectively), and Example 7 (measurement of β-galactosidase; page 54, lines 4-6). Examples 4 and 7 include labelled ACes in the methods exemplified.

The Examiner alleges that while the specification states that "incorporation of the analog label does not affect GFP protein expression," no data is provided to support this assertion, which is allegedly "at odds with the teachings of the prior art." The Examiner asserts that it is therefore unclear as to whether this is a statement of fact or a prophetic statement.

It is respectfully submitted that the filing of a patent application serves as conception and constructive reduction to practice of the subject matter described and claimed in the application ((MPEP §2138.05; *Hyatt v. Boone*, 146 F.3d 1348, 1352, 47 USPQ2d 1128, 1130 (Fed. Cir. 1998)). Further, no working examples or supportive data need be provided in the application. Rather, the requirements of § 112, first paragraph can be fulfilled by broad terminology. *In re Marzocchi et al.*, 469 USPQ 367 (CCPA 1971). As stated above, statements and disclosure in an application are presumed true:

> [a]s a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and describing the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.
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> . . . it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure (*In re Marzocchi & Horton*, 58 CCPA 1069, 439 F. 2d 220, 169 USPQ 367, 369-370 (1971)).

Furthermore, as discussed below, the "teachings of the prior art" alleged by the Examiner to be "at odds" with the instantly claimed subject matter bear little relevance to enablement of the steps of the instant methods as of their

filing date, and the Examiner has provided no basis to doubt the veracity of what is described and claimed.

As discussed above, Applicant is not required to provide data or illustrative examples in support of every assertion in the specification or everything within the scope of a broad claim (*In re Anderson*, 176 USPQ 331, at 333 (CCPA 1973)). Nonetheless, as discussed below, the accompanying DECLARATION under 37 C.F.R. § 1.132 of Sandra Vanderbyl demonstrates that by following the steps of the instant methods, one of skill in the art can obtain gene expression from labelled large nucleic acid molecules delivered into cells at an efficiency that is comparable to that of unlabelled DNA.

Thus, the specification teaches one skilled in the art how to introduce labelled large nucleic acid molecules into cells, how to measure delivery of the labelled nucleic acid molecules to cells by detecting labelled cells and how to measure gene expression from the labelled nucleic acid molecules in cells by detecting the product of a reporter gene.

Knowledge of those of skill in the art

At the time of filing of the application, a broad body of knowledge had amassed in the areas of delivery of nucleic acids to cells and in the use of reporter genes. Numerous such procedures are referenced in the instant application:

Agents to aid in the delivery of nucleic acid molecules and sources therefor are described in the specification. For example, reference is made to electroporation methods and apparatuses *e.g.*, in U.S. Patent Nos. 6,027,488, 5,993,434, 5,944,710, 5,507,724, 5,501,662, 5,389,069, 5,318,515 (page 23, lines 20-26). Procedures and methods for ultrasound systems also are provided. These include systems described in International PCT application No. WO 99/21584 and U.S. Patent No. 5,676,151 (page 22, line 10 through page 23, line 2). Additional delivery agents that can be used in the methods provided in this applicaiton. These include well-known agents, such as cationic reagents

such as Lipofectin, LipofectAMINE, and LipofectAMINE PLUS from Life Technologies, Inc., (Burlington, Ont., *see* U.S. Patent No. 5,334,761 and 5,736,392; *see, also* U.S. Patent No. 6,051,429), Effectene (Qiagen, Inc., Mississauga, Ontario) (Non liposomal lipid formulation), Metafectene (Biontex, Munich, Germany) (Polycationic lipid), Eu-fectins (Promega Biosciences, Inc., San Luis Obispo, CA) and numerous other cationic lipids (page 20, line 24 through page 21, line 18), as well as non-lipid compounds such as SUPERFECT™ (Activated dendrimer (cationic polymer:charged amino groups); Qiagen, Inc., Mississauga, ON) and CLONfectin™ (Cationic amphiphile N-t-butyl-N'-tetradecyl-3-tetradecyl-aminopropionamidine; Clontech, Palo Alto, CA) (page 21, line 20 through page 22, line 4).

Procedures relating to the use of reporter genes also are referenced and described in the instant application. For example, references are cited for reporters such as fluorescent protein, CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), *Nature 282*: 864-869) luciferase, *e.g.*, firefly luciferase (deWet *et al.* (1987), *Mol. Cell. Biol. 7*: 725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS 1*: 4154-4158; Baldwin *et al.* (1984), *Biochemistry 23*: 3663-3667); and alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem. 182*: 231-238, Hall *et al.* (1983), *J. Mol. Appl. Gen. 2*: 101) (page 16, lines 12-23).

Additionally, agents for detection of labelled nucleic acids such as BrdU-labelled DNA were known. The specification describes such, including, for example, Pittman *et al.*, *J Immunol Methods 103*: 87-92 (1987) (page 28, lines 16-20) and Gratzer *et al.* Cytometry (1981) 6:385-393 (page 55, lines 8-9).

These references to numerous published protocols for the labelling of DNA and detection thereof, agents for delivery of DNA into cells that can be used in the instantly claimed and disclosed methods, and analysis thereof for expression of encoded genes, including reporter gene products, demonstrate the large volume of information regarding tested and reliable agents available at the

time of filing of the instant application and thus evidence the advanced state of the art at the relevant time. The instant application details their application to the claimed and disclosed methods.

Presence of Working Examples

The specification provides numerous working examples and descriptions of the labelling, delivery and detection of delivered nucleic acid, including nucleic acid encoding a reporter gene and the measurement of expression of the reporter gene product (see discussion above). As detailed above, Examples 1, 4, 5, 6, and 7 of the application describe DNA delivery, measurement of labelled cells and measurement of reporter gene activity. Example 1, section B, provides details for the delivery of large nucleic acid molecules (page 40, line 4 through page 41, line 20) and for measuring gene expression from the delivered large nucleic acid molecules (page 41, lines 7-23). Example 4 exemplifies delivery of large labelled DNAs and the detection of labelled cells (page 47, line 22 through page 48, line 4). Example 5 provides detailed methods for the delivery of large nucleic acid molecules and measurement of GFP reporter gene expression from the delivered nucleic acids (page 48, line 15 through page 49, line 31). Example 6 exemplifies the delivery of large nucleic acid molecules to a variety of cell types and the measurement of the encoded GFP reporter gene expression (page 50, line 1 through page 51, line 4). Example 7 provides detailed methods for labeling of large nucleic acids (page 52, lines 12-31), delivery of large labelled nucleic acid molecules to cells using a variety of delivery agents (page 53, line 15 through page 54, line 4 and also Table 1 pages 54-55), the measurement of β-galactosidase activity after delivery (page 54, lines 4-6) and the detection of labeled cells to measure DNA delivery (page 55, line 8 through page 56, line 5 and also Table 2, pages 56-57).

Predictability

As discussed above and as known to those of skill in the art, the level of knowledge and skill in the delivery of DNA and the use of reporter genes for expression in cells as claimed in the instant application was high as of the effective filing date.

The pending claims are directed to methods in which labelled large nucleic acid molecules are delivered to cells, delivery is measured by detecting labelled cells and the detection of a reporter gene product is assessed as a measurement of gene expression. Having described the claimed methods, and detailed the numerous agents and procedures for effecting each step in the methods in the application, there is no issue of predictability in the instant case.

The Office Action alleges that the specification is not enabling for the claimed subject matter because the state of the art of obtaining expression from a labelled nucleic acid molecule at the time the instant application was filed was "highly unpredictable" and Applicant allegedly provides insufficient data to rebut this state of the art. To support the assertion, the Office Action cites Felgner *et al.* ((1999) WO 99/13719), Neves *et al.* (1999/2000) *Bioconjugate Chem.* 11:51-55), and Zelphati *et al.*, (1999) *Hum. Gene Therap.* 10:15-24. Each of these references is discussed in turn below.

Felgner *et al.*

The Office Action alleges that Felgner *et al.* ((1999) WO 99/13719) teaches that standard methods of labeling DNA using fluorescently tagged nucleotides do not allow detection of structurally and functionally intact plasmid in a real-time fashion in viable cells (paragraph bridging pages 1 and 2). The Office Action further points to a passage in Felgner *et al.* that states in its entirety: "All of the technologies discussed above for chemically modifying plasmid DNA result in DNA damage and interfere with its transcriptional activity" (page 2, line 36 through page 3, line 2).

First, it is respectfully submitted that any broad sweeping assertions as to general difficulties of detecting and/or obtaining expression from labelled DNA made in Felgner *et al.*, published in 1999, bears no relevance to the issue of whether the instant application is enabling as of the filing date, March 21, 2001, nor whether the instant application is enabled for the methods it teaches and claims. The citations pointed to by the Office Action only refer to technologies that Felgner *et al.* appraised as of 1999 and only a selected set of methods pertaining to plasmid DNA labeling. Felgner *et al.* does not provide any methods of detecting labelled large nucleic acid molecules, measuring gene expression from labelled large nucleic acid molecules or any limitations pertaining to such methods.

As discussed above, the instant application defines large nucleic acid molecules as being at least about 0.5 megabases in size (*see, e.g.*, page 9, line 30 through page 10, line 16). Felgner *et al.* provides no insights regarding delivery or expression of labelled nucleic acid molecules in this size range. Furthermore, Felgner *et al.* does not have the benefit of the teachings of the instant application

The instant application provides methods for labeling large nucleic acid molecules, delivering the labelled nucleic acid to cells and measuring gene expression as well as delivery (see, for example, Example 7, pages 52-57). The specification further teaches methods of labeling that do not result in the breakdown of the nucleic acids and additionally, the working examples provide a description of reagents that may be used to help stabilize labelled nucleic acids if necessary, such as hexylene glycol, spermine and spermidine (for example, see in Example 7, page 52, lines 29-31). Thus, the specification teaches one of skill in the art how to label large nucleic acids that can be delivered to cells to measure delivery and gene expression and additionally teaches methods to overcome the any limitations described by Felgner *et al.*

Second, the citation referred to by the Examiner from Felgner *et al.* reads in its entirety as:

> Other fluorescent labeling methods which utilize nick translation or photoaffinity labeling result in chemical breakdown of the starting material and thus any observations made with these materials may not be representative of the behavior of the original intact plasmid. None of the technologies presented above allow direct detection of structurally and functionally intact plasmid in a real-time fashion in viable cells (Felgner *et al.*, page 1, line 35 through page 2, line 3).

It is respectfully submitted that this citation has no bearing on the predictability of the methods as instantly claimed for introducing labelled large nucleic acid molecules into a cell and measuring their delivery and encoded gene expression. The methods alluded to by Felgner *et al.* refer to specific methods with specific labels that the author alleges cause breakdown of a particular type of DNA, plasmid DNA. Unlike the instant application, Felgner *et al.* does not provide any teachings for the preparation of labelled large nucleic acid molecules, measuring gene expression or delivery of labelled large nucleic acid molecules or overcoming limitations pertaining to such methods.

Neves *et al.*

The Office Action alleges that Neves *et al.* demonstrates that reporter gene expression was greatly reduced by labelling DNA with *p*-azido-tetrafluorbenzylamido-lissamine and abolished by labelling DNA with rhodamine labelled nucleotides. As discussed above, the assumption by the Examiner that the method of Neves *et al.*, first published in 1999, represents the state of the art of gene expression from labelled DNA as of the filing date of the instant application, March 21, 2001, is unfounded.

Neves *et al.* does not discuss general methods of nucleic acid labeling but in fact uses one specific method of labeling, photo-activated labeling, of a specific type of DNA, plasmid DNA. Further, the authors clearly point out that ultraviolet light used to photo-activate labeling induces DNA damage (page 54, second column, paragraph 3). Neves *et al.* also provides that damaged DNA

such as relaxed or linearized plasmid (page 51, paragraph bridging columns 1 and 2) leading to UV damage of the supercoiled plasmid DNA structure, whereas the instant application teaches methods for measuring gene expression and delivery of large nucleic acid molecules. Neves *et al.* suggests that "inactivation of the plasmid...could be explained by the high number of fluorescent nucleotides incorporated (250 per plasmid molecule) and by the modification of plasmid structure." (Page 54, col. 2, paragraph 4). This statement refers specifically to the the plasmid DNA molecules disclosed in Neves *et al.*, which are small and have distinct structural features relative to large labelled nucleic acid molecules, such as chromosomes, that are elements of the instantly claimed methods.

Neves *et al.*, like Felgner *et al.*, focusses on the limitations of photo-activated labelling of plasmid DNA, not the labelling of large nucleic acid molecules per se. Further, unlike Neves *et al.*, the instant application provides remedies to prevent delivery of damaged DNA and methods to increase nucleic acid stability and intactness (for example, page 33, line 31 through page 34, line 13 and page 52, lines 29-31). The teachings of the instant application recognize the manipulations of DNA that would result in instability or damage, and provide ways to overcome such difficulties. Thus, in addition to the irrelevancy of the general statements in Neves *et al.* to enablement of the instantly claimed methods as of the filing date, the instant application provides remedies for any deficiencies presented by Neves *et al.*.

The Office Action further alleges that Neves *et al.* teaches that the poor expression obtained with labelled vectors might result from interference with the transcription apparatus or sequestration or degradation of the labelled molecules. Again, Applicant respectfully points out that Neves *et al.* does not represent the state of the art as of the filing date of the instant application, nor does it represent a broad treatise of gene expression from labelled DNA.

Further, Neves *et al.* does not have the benefit of the teachings of the instant application.

Neves *et al.* pertains to photo-activated labelling of plasmid DNA of plasmid DNA (page 51, paragraph bridging columns 1 and 2) leading to UV damage of the supercoiled plasmid DNA structure, whereas the instant application teaches methods for measuring gene expression and delivery of large nucleic acid molecules. Neves *et al.* suggests that "inactivation of the plasmid...could be explained by the high number of fluorescent nucleotides incorporated (250 per plasmid molecule) and by the modification of plasmid structure." (Page 54, col. 2, paragraph 4). This statement refers specifically to the the plasmid DNA molecules disclosed in Neves *et al.*, which are small and have distinct structural features relative to large labelled nucleic acid molecules, such as chromosomes, that are elements of the instantly claimed methods.

Similarly, the statement of Neves *et al.* that plasmid-lissamine molecules could be sequestered or degraded in the cytoplasm (page 55, col. 1, first paragraph) has no bearing on the methods of the instant application, which are directed to delivery of labelled large nucleic acid molecules. This statement of Neves *et al.* is in specific reference to microinjecting plasmid-lissamine complexes into the cytoplasm. The instant application teaches numerous delivery agents and delivery methods (page 19, line 8 through page 23, line 26 and see also Examples 4, 5, 6 and 7, pages 46-57) that can be used in a method of delivering labelled large nucleic acid molecules and measuring gene expression and delivery.

Zelphati *et al.*

The Office Action cites Zelphati *et al.* ((1999) *Hum. Gene Therap. 10*: 15-24) as an example of the unpredictability of obtaining expression from a nucleic acid molecule that has been chemically modified to incorporate a label. The Office Action points to a passage in Zelphati *et al.*, which states:

> the methods that have been employed to directly modify DNA either reduce or destroy its ability to be transcribed. In addition, the available approaches to chemically modify plasmid, which utilize photolysis, nick translation, or the use of chemically active nucleotides analogs, attack the DNA randomly so that the final product is chemically heterogeneous and poorly defined.

First, although Zelphati *et al.* points out possible pitfalls of using labelled DNA in obtaining gene expression, these citations from 1999 do not represent the state of the art as of 2001, nor do they suggest that methods of the instant application, filed in 2001, are not enabling.

As with the other citations, Zelphati *et al.* does not have the benefit of the teachings of the instant application. The instant application teaches methods of delivering labelled large nucleic acid molecules and the measurement of gene expression and delivery of these nucleic acids. As discussed above, the large nucleic acid molecules of the instant application are at least about 0.5 megabases in size, and Zelphati *et al.* provides no teaching as to labeling, delivery or expression of nucleic acid molecules in this size range.

Further, the instant application provides ways to overcome the pitfalls referred to in Zelphati *et al.* Specifically, as discussed above, the instant specification teaches one skilled in the art that stability of the nucleic acid molecules to be delivered is assessed, for example by microscopy, after incorporation of label (page 33, line 31 through page 34, line 13); methods are provided for labelling nucleic acid molecules in ways that do not result in instability; and the working examples provide descriptions of reagents that may be used to stabilize labelled nucleic acids such as hexylene glycol, spermine and spermidine (for example see Example 7, page 52, lines 29-31). Thus, the specification teaches one of skill in the art how to make and use the instantly claimed subject matter, including ways to overcome the pitfalls and limitations discussed in Zelphati *et al.*

Second, it is respectfully submitted that a selective reading of Zelphati *et al.*, in which statements regarding the state of nucleic acid labeling and gene expression at the time of filing of the instant application are taken out of context, has resulted in a mischaracterization of the reference. While Zelphati *et al.* may summarize pitfalls of particular techniques as they pertain to plasmid DNAs, the authors then go on to provide a peptide nucleic acid hybridization labelling method for small plasmid DNA molecules that permits gene expression at a level similar to unmodified plasmid DNA.

It appears that the Office Action, in asserting the unpredictability of the art of obtaining gene expression from labelled nucleic acid molecules, has equated "limitations" with "unpredictability." It is respectfully submitted that although certain methods of labeling nucleic acid molecules can be associated with certain limitations in gene expression, this does not establish the art as unpredictable. The issue of whether the specific instant claims are enabled by the specification should not turn on specific limitations previously encountered in measuring delivery and expression of labelled nucleic acids. Rather, the relevant question with regard to enablement of the subject matter of the instant claims is whether the particular steps and materials of the claimed methods are described in the specification in such a way as to enable one skilled in the art to make and use the subject matter **as claimed**. Applicant is not aware of any requirement under current U.S. patent law specifying particular minimum levels of optimization and certified efficacy in order for a treatment-related area of art to qualify as sufficiently "predictable" such that lack of enablement under 35 U.S.C. § 112, first paragraph, is not a consideration. The relevant standard is not that of an established, fully optimized, method; rather, even in an *unpredictable* art, a patent application satisfies the requirements of 35 U.S.C. § 112, first paragraph, as long as it provides sufficient disclosure, either through illustrative examples or terminology, to teach those of skill in the art how to make and use the claimed subject matter without undue, experimentation.

Further, as discussed above, the teachings of the instant application clearly point to the limitations of the art cited by the Examiner, and provide ways to overcome them. Therefore, it is respectfully submitted that even if the art of the use of labelled nucleic acid molecules in delivery and expression systems was not a routine practice at the effective filing date of the subject application and was subject to limitations in its applicability, since the instant application provides solutions to any such pitfalls, such art should not qualify as a major factor in the determination of whether the requirements of 35 U.S.C. § 112, first paragraph, are satisfied with respect to the instantly claimed subject matter.

2. CONCLUSION

In light of the extensive teachings and examples in the specification, the high level of skill of those in this art, the knowledge of those of skill in the art, the fact that it is predictable to obtain gene expression from labelled nucleic acid molecules, and the breadth of the claims, it would not require undue experimentation for one of skill in the art to follow the steps of the instant methods to introduce labelled large nucleic acid molecules and measure delivery and gene expression thereof in a cell.

Accordingly, a consideration of the factors enumerated in *Ex parte Forman* leads to the conclusion that, based on the disclosure in the specification, undue experimentation would not be required to introduce labelled large nucleic acid molecules into a cell, measure labelled cells as an indication of delivery of nucleic acid and measure the product of a gene encoded by the labelled large nucleic acid as an indication of gene expression in a cell, as instantly claimed.

3. DECLARATION

Notwithstanding the above arguments, to evidence that the methods as claimed operate as claimed, attached is a DECLARATION under 37 C.F.R. §1.132 of Sandra Vanderbyl. The DECLARATION shows that when using the teachings of the application, large labelled nucleic acid is delivered and expressed as claimed.

Also, although this DECLARATION is submitted to rebut the Examiner's assertions of inoperativeness, it also further evidences enablement. It is noted that the level of skill in the biotechnical arts is recognized to be high (see, *e.g.*, *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986). Further, methods for performing the various steps of the claimed methods, such as labelling nucleic acids, delivering nucleic acids to cells and measuring delivery of and gene expression from the delivered nucleic acids are known to the skilled artisan.

Although Ms. Vanderbyl is an inventor of this application, in performing the experiments in the DECLARATION, she followed the teachings in the application. Since those of skill in this art typically have advanced degrees, Ms. Vanderbyl, who has an M.S. degree, is representative of a person of skill in this art with respect to performing experiments in accord with a disclosed protocol.

The DECLARATION demonstrates that using the procedures as taught in the above-captioned application delivery and expression of labelled large nucleic acid molecules in cells is achieved. The results demonstrate that delivery and expression of labelled large nucleic acid molecules in cells is similar to that of unlabelled nucleic acids.

Specifically, the DECLARATION of Vanderbyl, which incorporates the reference Vanderbyl *et al.*, *Cytometry*, 44:100-105 (2001), demonstrates that large nucleic acid molecules, exemplified using artificial chromosomes, can be (i) labelled with either iododeoxyuridine (IdU) or bromodeoxyuridine (BrdU); (ii) delivered into a host cell by transfection methods using such reagents as

Superfect and LipofectAMINE 2000 (iii) detected by monitoring the labelled cells using an IdU/BrdU antibody and flow cytometry; and (iv) measured for expression of a reporter gene such as green fluorescent protein (GFP) expressed from the labelled nucleic acid molecule in the cell. The DECLARATION further demonstrates that by following the teachings of the specification, one of skill in the art can obtain gene expression of a product encoded by a labelled large nucleic acid at an efficiency that is comparable to that of an unlabelled large nucleic acid under similar conditions.

The DECLARATION describes the labeling of cell lines CHO E42019 and GFP A9, each containing an artificial chromosome of about 120 megabases and 90 megabases, respectively. Labeling is performed with either IdU or with BrdU. Cells are labelled with a range of label concentrations and with continuous and pulse label methods. The DECLARATION demonstrates the use of flow cytometry as well as microscopy to detect labelled cells. Further, the DECLARATION demonstrates that GFP is expressed from cells containing the labelled large nucleic acid, and that this expression (an average efficiency of 47.3%) does not differ significantly from the expression of GFP in unlabeled cells (an average efficiency of 52.8%).

The results of these analyses demonstrate that the methods described in the above-referenced application can be used to monitor labelled large nucleic acid delivery and gene expression of products encoded by the large nucleic acid. Additionally, the DECLARATION demonstrates that in following the steps of the instantly claimed methods as provided in the specification, labeling of large nucleic acids does not affect the expression of encoded gene products as compared to gene expression from unlabelled large nucleic acids.

Therefore, the DECLARATION provides further evidence that the disclosure in the above-captioned application enables methods for delivering labelled large nucleic acid molecules, measuring labelled cells as an indication of delivery of nucleic acid and measuring the product of a reporter gene expressed

from a labelled large nucleic acid as an indication of gene expression in a cell. Vanderbyl *et al.*, hence, is relied upon, not to establish enablement, but to demonstrate that labelled large nucleic acids can be readily made, delivered to cells, detected in cells and expressed in cells by following the teachings in the specification. The DECLARATION demonstrates element-for-element and step-for-step that, by following the teaching in the application, one can: (i) prepare and deliver labelled large nucleic acids to cells; (ii) measure nucleic acid delivery by measuring labelled cells; (iii) measure expression of a gene product encoded by the labelled large nucleic acid; and (iv) obtain gene expression from a labelled large nucleic acid at a level that is comparable to expression from an unlabelled large nucleic acid. Accordingly, Applicant respectfully submits that the claims are commensurate in scope with the Applicant's discovery and its disclosure within the above-captioned application.

THE REJECTION OF CLAIM 9 UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claim 9 is rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter. Specifically, the Office Action alleges that the reference to "step(a)" in line 1 of the claim has no antecedent basis in Claims 1 and 6, from which Claim 9 depends. Reconsideration of this rejection is respectfully requested in view of the amendments herein and the following remarks.

Claim 9 is amended to more particularly point out the claimed subject matter. Claim 9 is amended to refer to the step of "introducing the labelled large nucleic acid molecules", which finds antecedent basis in Claim 1, upon which Claim 6 and therefore Claim 9 depends. Basis for "introducing labelled large nucleic acid molecules" with a cationic delivery agent as recited in Claim 9 also is found in the instant application (for example, see page 20 line 4 through page 22, line 4 and also in the working examples, Table 1, pages 54-55). Therefore, Claim 9 as amended has antecedent basis in Claims 1 and 6 and is no longer indefinite.

REJECTION OF CLAIMS 1, 2, 4, 6-10, 12, 14-16 and 30 UNDER 35 U.S.C. § 102(b)

A. Claims 1, 2, 4, 6-10, 12, 14-16 and 30 are rejected under 35 U.S.C. § 102(b) as being anticipated by Felgner *et al.* (WO 99/13719 (1999)). The Office Action alleges that Felgner *et al.* discloses a method for detecting or determining delivery and expression of a nucleic acid introduced into a cell comprising: introducing labelled nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and measuring the product of the reporter gene. This rejection is respectfully traversed.

RELEVANT LAW

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration. In re Spada, 15 USPQ2d 1655 (Fed. Cir, 1990), In re Bond, 15 USPQ 1566 (Fed. Cir. 1990), Soundscriber Corp. v. U.S.. 360 F.2d 954, 148 USPQ 298, 301, adopted 149 USPQ 640 (Ct. Cl.) 1966. See, also, Richardson v. Suzuki Motor Co., 868 F.2d 1226, 1236, 9 USPQ2d 1913,1920 (Fed. Cir.), cert. denied, 110 S.Ct. 154 (1989). "[A]ll limitations in the claims must be found in the reference, since the claims measure the invention." In re Lang, 644 F.2d 856, 862, 209 USPQ 288, 293 (CCPA 1981). Moreover it is incumbent on Examiner to identify wherein each and every facet of the claimed invention is disclosed in the reference. Lindemann Maschinen-fabrik Gmbh v. American Hoist and Derrick Co., 730 F.2d 1452, 221 USPQ 481 (Fed. Cir. 1984). Further, the reference must describe the invention as claimed sufficiently to have placed a person of ordinary skill in the art in possession of the invention. An inherent property has to flow naturally from what is taught in a reference In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981).

"Rejections under 35 U.S.C. §102 are proper only when the claimed subject matter *is* identically disclosed or described in the "'prior art'" . . .the

[r]eference must clearly and unequivocally disclose the claimed compound or direct those skilled in the art to the compound without *any* need for picking, choosing, and combining various disclosures not directly related to each other by the teachings in the cited references. Such picking and choosing may be entirely proper when making a rejection of a 103, obviousness rejection, where the applicant must be afforded an opportunity to rebut with objective evidence any inference of obviousness which may arise from the *similarity* of the subject matter which he claims to the prior art, but it has no place in the making of a 102, anticipation rejection." (Emphasis in original). *In re Arkey, Eardly, and Long*, 455 F.2d 586, 172 USPQ 524 (CCPA, 1972).

THE CLAIMS

Claim 1 is directed to a method of nucleic acid delivery and measurement of nucleic acid delivery and gene expression. The steps of the method include:

> introducing labelled large nucleic acid molecules that encode a reporter gene into cells;
> detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and
> measuring the product of the reporter gene as an indication of DNA expression in the cell, whereby delivery and expression of nucleic acid molecules in the cell is detected or determined.

Dependent claims 2, 4, 6-10, 12, 14-16 and 30 specify particulars, such as methods of detection by flow cytometry, fluorometry, cell imaging or fluorescence spectroscopy (claim 2); the type of nucleic acid including DNA (claim 4) artificial chromosomes, plasmids, chromosome fragments, naked DNA or natural chromosomes (claim 12); the types of reporter genes encoding a fluorescent protein or enzyme or antibody (claims 6-8); agents for delivering nucleic acids (claims 9-10); and the types of cells (claims 14-16). Thus, all of the instantly claimed methods are directed to the introduction of labelled large nucleic acid molecules into a cell, detection of their delivery and measurement of expression of a reporter gene encoded by the labelled large nucleic acid molecules.

ANALYSIS

Differences between the claims and the disclosure of Felgner *et al.* (WO 99/13719)

Felgner *et al.* discloses methods for introducing peptide nucleic acid (PNA) plasmid DNA hybrids into cells. The plasmids are on the order of 10 kB, and hence are not large nucleic acids. Felgner *et al.* does not disclose methods for delivery and expression of large nucleic acid molecules.

In the method of Felgner, PNA is hybridized in a sequence-specific manner to the plasmid. In some embodiments of Felgner *et al.*, the PNA is conjugated to a label, exemplified by a rhodamine-PNA conjugate, to monitor plasmid biodistribution. The plasmid encodes a reporter gene, either GFP or β-galactosidase, to measure gene expression. Felgner *et al.* also discloses the use of fluorescence microscopy to monitor labelled cells. Felgner *et al.* discloses the use of cationic liposomes, specifically DOPE:DMRIE, as a delivery agent for plasmid introduction into cells. Felgner *et al.* discloses the use of animal cells such as mouse and monkey cells, specifically mouse melanoma and transformed Cos-7 cell lines, to deliver labelled plasmid DNA.

Felgner *et al.* does not disclose methods for the introduction of labelled large nucleic acids into cells, nor measurement of their delivery or expression of genes encoded by the large labelled nucleic acids.

Analysis

In contrast, claim 1 as amended herein and dependent claims 2, 4, 6-10, 12, 14-16 and 30 are directed to introducing labelled large nucleic acid molecules encoding a gene into cells, and the measurement of nucleic acid delivery and gene expression.

As defined in the specification, large nucleic acids are "at least about 0.5 megabases" (*i.e.*, about 500 kB) (page 9, line 30 through page 10, line 1).

The plasmid DNA molecules diclosed in Felgner *et al.* are of the order of less than 10kB (*see* Example 1 beginning at page 12 and references incorporated therein).

Felgner *et al.* does not disclose methods using labelled large nucleic acids, such as chromosomes, where delivery of the labelled large nucleic acid is detected and expression of genes(s) encoded by the labelled large nucleic acid is measured. All of the rejected claims (Claim 1 and claims dependent thereon), on the other hand, are directed to delivery and expression of labelled large nucleic acids in cells.

Therefore, since anticipation requires that a reference disclose all elements as claimed, Felgner *et al.*, which does not disclose the introduction of a labelled large nucleic acid into a cell, does not anticipate any of the rejected claims 1, 2, 4, 6-10, 12, 14-16 and 30, all of which are directed to methods of introducing labelled large nucleic acids into cells and measuring nucleic acid delivery and gene expression.

B. Claims 1, 2, 4, 6, 7, 9, 10, 12, 14-16 and 30 are rejected under 35 U.S.C. § 102(b) as being anticipated by Neves *et al.* ((1999/2000) *Bioconjugate Chem.* 11: 51-55) because Neves *et al.* teaches a method for introducing labelled nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid onto a cell; and measuring the product of a reporter gene. This rejection is respectfully traversed.

RELEVANT LAW

See above.

CLAIMS

See above.

ANALYSIS

Differences between the claims and the disclosure of Neves *et al.* ((1999/2000) *Bioconjugate Chem. 11*: 51-55)

Neves *et al.* discloses the labeling of plasmid DNA with p-azido-tetrafluorobenzylamido-lissamine and with rhodamine nucleotides. Neves *et al.* discloses the introduction of these labelled plasmids and the detection of labelled cells by fluorescence microscopy. Neves *et al.* discloses that their methods of introducing plasmids into cells results in detection of reduced reporter gene (β-galactosidase) activity from labelled DNA as compared with unlabelled DNA (Figure 2, p. 53).

Neves *et al.* does not disclose a method of introducing labelled large nucleic acids into cells, nor measuring delivery of or gene expression from the delivered labelled large nucleic acids. The plasmids labelled by the method of Neves *et al.* are only about 7 kb (*see* Figure 1, page 53), whereas, as discussed above, large nucleic acid molecules as defined in the instant application are "at least about 0.5 megabases" or 500kB (page 9, line 30 through page 10, line 1). Neves *et al.* does not disclose the introduction of labelled nucleic acid molecules that are artificial chromosomes, chromosome fragments or natural chromosomes into cells. Therefore, the plasmids disclosed in Neves *et al.* are several orders of magnitude smaller than the large nucleic acid molecules of the instant application.

Therefore, since anticipation requires that a reference disclose all elements as claimed, Neves *et al.*, which does not disclose the introduction of a labelled large nucleic acid into a cell, nor its detection or measurement of expression of gene(s) encoded by the large nucleic acid, Neves *et al.* does not anticipate any of the rejected claims, all of which specify these elements.

C. **Claims 1, 2, 4, 6, 8-10, 12, 14-16 and 30** are rejected under 35 U.S.C. § 102(b) as being anticipated by Zelphati *et al.* (1999) *Hum. Gene Therap. 10*: 15-24 because Zelphati *et al.* discloses a method for introducing labelled nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid onto a cell; and measuring the product of a reporter gene. This rejection is respectfully traversed.

RELEVANT LAW

See above.

CLAIMS

See above.

ANALYSIS

Differences between the claims and the disclosure of Zelphati *et al.* (1999) *Hum. Gene Therap. 10*: 15-24 ("Zelphati *et al.*")

Zelphati *et al.* discloses a method of hybridizing rhodamine-labelled peptide nucleic acid molecules (PNA) to plasmid DNA encoding a green fluorescent protein (GFP) reporter gene and the visualization of the labelled plasmid and GFP in cells. Zelphati *et al.* does not disclose the introduction of labelled large nucleic acids into cells. The plasmids used in Zelphati *et al.* range in size from about 5 kB to about 8 kB (page 2, column 2, first paragraph). On the other hand, as discussed above, the "large" nucleic acids that are elements of the instant claims are at least about 500 kB in size (page 9, line 30 through page 10, line 1).

Therefore, since anticipation requires that a reference disclose all elements as claimed, Zelphati *et al.*, which does not disclose the introduction of a labelled large nucleic acid into cells, nor the monitoring of nucleic acid delivery and gene expression of products encoded by the labelled large nucleic acid, does not anticipate any of the rejected claims 1, 2, 4, 6-10, 12, 14-16 and 30, all of which specify these elements.

REJECTION OF CLAIMS 1-3 AND 11-13 UNDER 35 U.S.C. §103(a)

Claims 1-3 and 11-13 are rejected under 35 U.S.C. §103(a) as being unpatentable over either one of Felgner *et al.* (WO 99/13719 (1999)) or Zelphati *et al.* (*Hum. Gene Therap. 10*: 15-24 (1999)) in view of Nolan *et al.* (WO 00/34436 (2000)). The Office Actionu alleges that Felgner *et al.* and Zelphati *et al.* teach methods for the detection or determination of delivery and expression of a labelled nucleic acid introduced into a cell including the steps of: introducing labelled nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid into the cell; and measuring the product of the reporter gene.

The Office Action acknowledges that Felgner *et al.* and Zelphati *et al.* do not teach the use of flow cytometry to detect the labelled cells , nor the delivery of large DNA or chromosomes, but urges that Nolan *et al.* cures these deficiencies. The Office Action alleges that Nolan *et al.* teaches a method of delivering DNA into cells wherein cells that have taken up the DNA are detected by flow cytometry. It is further alleged that Nolan *et al.* teaches a method for the delivery of fluorescently labelled large DNA molecules into cells where the large DNA molecule includes natural chromosomes and artificial chromosomes.

With respect to delivery of nucleic acids into cells, the Office Action concludes that it would have been obvious to one of skill in the art to modify the methods taught by Felgner *et al.* and Zelphati *et al.* to include the separation of the labelled cells by flow cytometry as allegedly suggested by Nolan *et al.* The Office Action supports this assertion by stating that one of skill in the art would have been motivated to combine the teachings of Felgner *et al.* or Zelphati *et al.* and those of Nolan *et al.*, because Nolan *et al.* states that "flow cytometry methods such as fluorescence-activated cell sorting (FACS) are ideal tools to employ in chromosome insertion methods due to their ability to rapidly process and analyze large numbers of individual cells"; therefore, absent evidence to the contrary, one of skill in the art would have had a reasonable

expectation of success in using the methods of Nolan *et al.* to deliver any fluorescently labelled nucleic acid.

With respect to obtaining gene expression from labelled DNA introduced into cells, the Office Action concludes that it would have been obvious to one of skill in the art to modify the methods taught by Felgner *et al.* and Zelphati *et al.* according to the teachings of Nolan *et al.* to deliver fluorescently labelled transcriptionally active chromosomes into cells. The Office Action supports this assertion by stating that one of skill in the art would have been motivated to combine the teachings of Felgner *et al.* or Zelphati *et al.* and those of Nolan *et al.* because Nolan *et al.* states that "the introduction of intact single chromosomes into cells offers unprecedented usefulness as a...method for generating transgenic animals" and Zelphati *et al.* teaches that their methodology provides a means to tag DNA without disrupting the structural or functional integrity of the DNA. Therefore, it is alleged that absent evidence to the contrary, one of skill in the art would have had a reasonable expectation of success in using the labeling methods of Felgner *et al.* and Zelphati *et al.* to deliver any nucleic acid molecule.

This rejection is respectfully traversed.

RELEVANT LAW

In order to set forth a *prima facie* case of obviousness under 35 U.S.C. §103: (1) there must be some teaching, suggestion or incentive supporting the combination of cited references to produce the claimed invention (ACS Hospital Systems, Inc. v. Montefiore Hospital, 732 F.2d 1572, 1577, 221 USPQ 329, 933 (Fed. Cir. 1984)) and (2) the combination of the cited references must actually teach or suggest the claimed invention. Further, that which is within the capabilities of one skilled in the art is not synonymous with that which is obvious. Ex parte Gerlach, 212 USPQ 471 (Bd. APP. 1980). Obviousness is tested by "what the combined teachings of the references would have suggested to those of ordinary skill in the art." In re Keller, 642 F.2d 413, 425,

208 USPQ 871, 881 (CCPA 1981), but it cannot be established by combining the teachings of the prior art to produce the claimed subject matter, absent some teaching or suggestion supporting the combination (ACS Hosp. Systems, Inc. v Montefiore Hosp. 732 F.2d 1572, 1577. 221 USPQ 329, 933 (Fed. Cir. 1984)). "To imbue one of ordinary skill in the art with knowledge of the invention in suit, when no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher" W.L. Gore & Associates, Inc. v. Garlock Inc., 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983).

CLAIMS

As described above, claims 1-3 and 11-13 are directed to methods of introducing labelled large nucleic acids encoding a reporter gene into cells and measuring DNA delivery and reporter gene expression. Claims 2 and 3 are directed to the use of flow cytometry, fluorometry, cell imaging or fluorescence spectroscopy in detecting labelled cells. Claims 11-13 further define the large nucleic acid as natural chromosomes, artificial chromosomes, plasmids, fragments of chromosomes and naked DNA greater than about 0.6 megabases in size.

Teachings of the cited art and the differences between the teachings of the cited art and claims 1-3 and 11-13

Felgner *et al.*

As discussed above, Felgner *et al.* teaches methods for introducing peptide nucleic acid (PNA), including rhodamine-PNA conjugates, hybridized in a sequence-specific manner to plasmid DNA into cells and measuring gene expression from these plasmids.

Felgner *et al.* does not teach or suggest methods for the delivery of large labelled nucleic acid molecules. As discussed above, the instant application

defines "large" nucleic acids as being "at least about 0.5 megabases" (*i.e.*, about 500 kB) (page 9, line 30 through page 10, line 1).

The plasmid DNA molecules taught by Felgner *et al.* are of the order of less than 10kB (*see* Example 1 beginning at page 12 and references incorporated therein). Felgner *et al.* does not teach any methods using labelled large nucleic acids, such as chromosomes, where delivery of the labelled large nucleic acid AND expression of genes(s) encoded by the labelled large nucleic acid are measured. Felgner *et al.* does not teach the introduction of labelled artificial chromosomes, chromosomes fragments or natural chromosomes into cells.

Zelphati *et al.*

As discussed above, Zelphati *et al.* teaches methods for hybridizing rhodamine-labelled peptide nucleic acid (PNA) to plasmid DNA encoding a reporter gene and the detection of the labelled plasmid and reporter gene products (GFP or β-galactosidase) in cells.

Zelphati *et al.* does not teach or suggest the introduction of large nucleic acids nor the introduction of large labelled nucleic acids. The plasmids used in Zelphati *et al.* range between about 5 kB and about 8 kB (page 2, column 2 first paragraph). In contrast, the large labelled nucleic acids used in the instantly claimed methods are at least about 500 kB (page 9, line 30 through page 10, line 1). Zelphati *et al.* does not teach the introduction of labelled artificial chromosomes, chromosome fragments or natural chromosomes into cells. Further, Zelphati *et al.* does not teach the introduction of labelled nucleic acids that are plasmids or naked DNA that are large nucleic acids, at least about 0.5 megabases (500kB) as defined by the instant application. As mentioned above, the plasmid DNA taught by Zelphati *et al.* is at least about two orders of magnitude smaller than the nucleic acids that are delivered by the instantly claimed methods.

Nolan *et al.*

Nolan *et al.* teaches methods and an apparatus that employ fluorescence activated cells sorting (FACS) to process large numbers of cells in a chromosome insertion protocol. Nolan *et al.* teaches subjecting a cell to a laser pulse light under conditions sufficient to form a transient hole in the cell plasma membrane, then introducing a single chromosome into the cell through the hole. Nolan *et al.* further teaches that fluorescence activated cells sorting (FACS), a type of flow cytometry, can be employed to confirm the insertion of a single chromosome within a cell.

Nolan *et al.* does not teach or suggest the introduction of labelled large nucleic acid molecules encoding a reporter gene into cells, nor measurement of reporter gene product as an indication of gene expression. The methods of Nolan *et al.* are focussed on the verification of single chromosome insertion. Nolan *et al.* mentions that the chromosome may be fluorescently labelled, but provides no teaching or suggestion for obtaining such labelled chromosomes. Further, Nolan *et al.* does not teach the detection of reporter gene expression in combination with measurement of nucleic acid delivery of labelled large nucleic acid molecules encoding the reporter gene.

ANALYSIS

The combination of teachings of Felgner *et al.* or Zelphati *et al.* with the teachings of Nolan *et al.* does not result in the instantly claimed methods.

None of the cited references, singly or in any combination, teaches or suggests a method for the introduction of labelled large nucleic acid molecules encoding a reporter gene and the detection of delivery and measurement of gene expression. As discussed above, neither Felgner *et al.* nor Zelphati *et al.* teaches or suggests methods for introducing labelled large nucleic acid molecules into cells and detecting their delivery and measuring their expression.

The methods of Felgner *et al.* and Zelphati *et al.* are directed to the monitoring delivery and expression of plasmid DNA that is under 10 kB in size.

Nolan *et al.*, which is directed to the insertion of chromosomes into cells, provides no teaching or suggestion regarding any method for delivery of large nucleic acid molecules nor for detecting or determining delivery and expression of such nucleic acid. Nolan *et al.* only provides an apparatus to deliver chromosomes into cells and a method to screen for and recover eukaryotic cells having a single chromosome inserted therein. Nolan *et al.* does not teach or suggest monitoring delivery and measuring expression of chromosomes in the cells. Therefore, Nolan *et al.* does not cure the deficiencies of either Felgner *et al.* or Zelphati *et al.*

The Office Action alleges that absent evidence to the contrary, one of ordinary skill in the art would have had a reasonable expectation of success in using the methods of Nolan *et al.* in combination with either Felgner *et al.* or Zelphati *et al.*to deliver <u>any</u> fluorescently labelled nucleic acid.

The claims of the present method are directed to steps of: introducing labelled large nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid into the cell; and measuring the product of the reporter gene. The combination of teachings of tghe references cited must provide all of these steps, not just the single step of introducing fluorescently labelled nucleic acid. Felgner *et al.* and Zelphati *et al.* do not provide any teaching or suggestion for delivery of large nucleic acids molecules nor monitoring/measuring delivery and expression of large labelled nucleic acids in cells. Nolan *et al.* is directed to single chromosome insertion and cell sorting to recover cells containing inserted chromosomes. Therefore, Nolan *et al.*, which also does not teach or suggest a method for monitoring/measuring delivery and expression of large labelled nucleic acids in cells, cannot cure the deficiencies of either Felgner *et al.* or Zelphati *et al.*

One of ordinary skill in the art would have no expectation of success from the combination of Felgner *et al.* or Zelphati *et al.* with Nolan *et al.* in measuring

the delivery and expression of labelled large nucleic acids encoding a reporter gene, *i.e.*, to provide all the steps of the instantly claimed methods. The Examiner asserts that one of ordinary skill in the art could use the delivery method of Nolan *et al.* in the method of Felgner *et al.* or Zelphati *et al.*; Nolan *et al.* does not, however, provide methods for the measurement of nucleic acid delivery and gene expression, nor any teaching or suggestion thereto. In fact, insertion of a single copy of fluorescently labelled chromosome as taught by Nolan et al. would be incompatible with the methods of either Zelphati *et al.* or Felgner *et al.*, which each teach delivery of several thousand copies of plasmid molecules to a cell in order to measure their delivery and expression.

For example, Zelphati *et al.* (which is a near identical if not identical method to that of Felgner *et al.*) states that an average of 30,000-50,000 copies of plasmid/cell were present using the method described therein (page 19, column 2, paragraph 2). There is no teaching or suggestion that the method of Zelphati *et al.*, designed to operate with many thousands of copies of plasmid to monitor gene expression, would be useful or workable with a lower number of nucleic acid molecules, such as the introduction of a single fluorescent chromosome into a cell as taught by Nolan *et al.* Neither the method of Felgner *et al.* nor that of Zelphati *et al.* teaches or suggests measurement of gene expression from such a low copy number of nucleic acid. Thus, one of ordinary skill in the art would have no reasonable expectation of success in combining the delivery methods of Nolan *et al.* with the teachings of Felgner *et al.* or Zelphati *et al.* to obtain and measure delivery and expression of a labelled large nucleic acid encoding a reporter gene.

The Office Action further alleges that it would have been obvious to one of skill in the art to modify the methods taught by Felgner *et al.* and Zelphati *et al.* according to the teachings of Nolan *et al.* to deliver fluorescently labelled transcriptionally active chromosomes into cells. The Office Action alleges that one of skill in the art would have been motivated to combine the teachings of

Felgner *et al.* or Zelphati *et al.* and those of Nolan *et al.* because Nolan *et al.* states that "the introduction of intact single chromosomes into cells offers unprecedented usefulness as a...method for generating transgenic animals" and Zelphati *et al.* teaches that their methodology provides a means to tag DNA without disrupting the structural or functional integrity of the DNA.

None of the cited references, singly or in any combination, teaches or suggests a method for the introduction of labelled large nucleic acid molecules and the determination or detection of delivery and gene expression where the introduced nucleic acid is a large nucleic acid or a chromosome as defined herein (about 0.5 megabases or greater, *see, e.g.*, page 9, line 30 through page 10, line 16). The Examiner asserts that the motivation to combine Zelphati *et al.* or Felgner *et al.* with Nolan *et al.* comes from a citation, quoted in the paragraph above, that is in fact a general statement on the utility of introducing chromosomes into cells; Nolan *et al.* does not teach or suggest anything about monitoring delivery of chromosomes and gene expression from chromosomes. Additionally, Nolan *et al.* suggests "there exists a need for a method to rapidly and reliably process cells in a manner that provides for the introduction of a single chromosome into a cell in a verifiable manner" (page 1, lines 25-27). Nolan *et al.* provides no teaching or suggestion that there is a need for monitoring gene expression nor measuring gene expression along with DNA delivery as set forth in the instantly claimed methods. Thus, contrary to the Examiner's assertion, there is no teaching or suggestion in Nolan *et al.*, which lacks any reference to measuring gene expression, that would cure the deficiencies in Felgner *et al.* or Zelphati *et al.*, neither of which provide any teaching or suggestion as to obtaining successful delivery and gene expression of large nucleic acid molecules.

The Office Action further alleges that absent evidence to the contrary, one of ordinary skill in the art would have had a reasonable expectation of

success in using the labeling methods of Felgner *et al.* and Zelphati *et al.* in combination with Nolan *et al.* to deliver any nucleic acid molecule.

The methods of Felgner *et al.* and Zelphati *et al.* provide only for labelling of small plasmid DNAs with peptide nucleic acids (PNAs). There is no teaching or suggestion in Felger *et al.* or Zelphati *et al.* that the labeling method is suitable for large nucleic acids or how PNA may be used with large nucleic acid molecules. Both references use plasmids on average 6-7 kilobases (no larger than 8.2 kilobases). "Large" nucleic acid as defined in the instant application is at least about 0.5 megabases (500 kilobases), at least two orders of magnitude larger than what is taught by Felgner *et al.* and Zelphati *et al.* Nolan *et al.* does not cure this deficiency, providing only that labelled chromosomes can be used with the described FACS apparatus. Nolan *et al.* does not teach or suggest how one obtains labelled large nucleic acids. It is only in the instant application that methods are provided for labeling large nucleic acid molecules such that the labelled large nucleic acids encoding a reporter gene can be delivered and expressed in cells with an efficiency comparable to that of unlabelled nucleic acids.

Furthermore, the labelling methods taught by Felgner *et al.* and Zelphati *et al.* are specially engineered to maintain the structural integrity of plasmid DNA. Neither Zelphati *et al.* nor Felgner *et al.* teaches or suggests methods for the labeling of large nucleic acids in a manner that maintains their transcriptional integrity. The methods taught by Felgner *et al.* and Zelphati *et al.* rely on sequence-specific hybridization of a peptide nucleic acid (PNA) with a plasmid to conjugate the label to the plasmid. Neither reference provides guidance on how one of ordinary skill in the art would engineer such conjugates in large nucleic acids, given the limitations described by both Felgner *et al.* and Zelphati *et al.*, for example, that PNAs can inhibit gene expression, block restriction enzyme activity and act as an artificial promoter (Felgner *et al.*, page 2, lines 21-25) and furthermore, in designing PNAs that "the probe itself should not have influence

on plasmid function" (Felgner *et al.*, page 2, lines 35-36). Additionally, Zelphati *et al.* states that PNAs are known to inhibit several metabolic processes such as translation, including ribosome translocation, transcription, including inhibition of RNA polymerase and telomerase activity (page 20, column 2, paragraph 3). Neither Felgner *et al.* nor Zelphati *et al.* teach or suggest how to overcome these limitations for the labeling of large nucleic acids such that function is not impaired or altered as a result of the PNA conjugation. Nolan *et al.*, which offers no teachings as to labelling large nucleic acids, much less labelling in a manner that preserves their transcriptional integrity, does not remedy the deficiencies of Felgner *et al.* and Zelphati *et al.* It is only in the instant application that a method is provided in which labelled large nucleic acids encoding a reporter gene that maintain their transcriptional integrity are introduced into cells, and their delivery and expression measured.

The rejection over the cited references is based on improper use of hindsight

The disclosure of the Applicant cannot be used to hunt through the prior art for the claimed elements and then combine them as claimed. *In re Laskowski*, 871 F.2d 115, 117, 10 USPQ2d 1397, 1398 (Fed. Cir. 1989). "To imbue one of ordinary skill in the art with knowledge of the invention in suit, when no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher." *W.L. Gore & Associates, Inc. v. Garlock Inc.*, 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983). It appears that the Examiner has combined the teachings of the cited art with those of the instant application.

Since the cited references do not teach or suggest any of the elements of the instantly claimed methods pointed out by the Examiner (introducing labelled large nucleic acid molecules encoding a reporter gene into a cell, detecting labelled cells as an indication of nucleic acid delivery, and measuring the product

of the reporter gene as an indication of DNA expression in the cell), as set forth in the instantly claimed methods, the only way to combine the references to result in the instantly claimed methods would require the use of the instant application as a guide to look for isolated elements of, *e.g.*, "DNA", "chromosome", "labeling", and "gene expression" and combine them to produce the instant claims. None of these elements are recited in the cited references in the context of obtaining and introducing labelled large nucleic acids into cells and measuring their delivery and expression. In fact, the only reference that mentions a "large" nucleic acid (chromosome), Nolan *et al.*, is not directed to any method of nucleic acid delivery and expression in cells at all; rather, it is aimed solely at inserting a single chromosome into cells, and cell-sorting (FACS) to retrieve cells containing a single inserted chromosome. Any labeling of the chromosome taught in Nolan *et al.* is in the context of cell-sorting, not to monitor delivery and expression. Therefore, it appears that the Examiner has isolated these elements from the cited references and incorporated them into the methods as instantly claimed.

Therefore, in addition to the fact that the combination of the cited references does not result in the instantly claimed subject matter, the Office has improperly employed hindsight in setting forth an alleged *prima facie* case of obviousness.

CONCLUSION

In summary, the combination of the teachings of Felgner *et al.* or Zelphati *et al.* with Nolan *et al.* does not result in the instantly claimed subject matter, the methods of which include steps of: introducing labelled large nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid into the cell; and measuring the product of the reporter gene. None of the references, singly or in any combination, teaches or suggests a method for detecting delivery and gene expression in cells that involves introducing labelled large nucleic acids encoding reporter genes

into cells, measuring labelled cells as an indication of delivery, and measuring reporter gene expression as an indication of DNA expression. Therefore, the Examiner has failed to set forth a *prima facie* case of obviousness.

* * *

In view of the above amendments and remarks, reconsideration and allowance of the application are respectfully requested.

Respectfully submitted,
HELLER EHRMAN WHITE & McAULIFFE LLP

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: DE JONG et al.

Serial No.:09/815,981 Group Art Unit: 1636

Filed: March 22, 2001 Examiner: Sullivan, Daniel M.

For: METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

ATTACHMENTS

The following attachments are provided in connection with the Amendment filed herewith.

1. Marked up paragraph and claims pursuant to 37 C.F.R. §1.121.
2. A DECLARATION of VANDERBYL pursuant to 37 C.F.R. §1.132.
3. A copy of the following publication:

 Vanderbyl *et al.*(2001) *Cytometry 44*:100-105

OIPE JC81 JUN 16 2003 PATENT & TRADEMARK

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: de Jong *et al.*
Serial No.: 09/815,981
Filed: March 22, 2001
Conf. No.: 7622
Cust. No.: 24961
For: *METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF*
Art Unit: 1636
Examiner: Sullivan, D.M.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
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I hereby certify that this paper and the attached papers are being deposited with the United States Postal "Express Mail Post Office to Addressee" Service under 37 C.F.R. §1.10 on the date indicated above and addressed to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

06/16/2003
Date

Debra Walker

MARKED UP PARAGRAPHS AND CLAIMS (37 C.F.R. § 1.121)

IN THE SPECIFICATION:

Please amend the paragraph on page 1, lines 14-23, with the following:

A number of methods of delivering nucleic acid molecules, particularly plasmid DNA and other small fragments of nucleic acid, into cells have been developed. These methods are not ideal for delivery of larger nucleic acid molecules. Thus, there is a need for methods of delivering nucleic acid molecules of increasing size and complexity, such as artificial chromosomes, into cells. Methods are required for use with *in vitro* and *in vivo* procedures such as gene therapy and for production of transgenic animals and [pants] plants. Furthermore, there is a need for the ability to rapidly and simply determine and assess the efficiency of delivery of DNA into cells.

IN THE CLAIMS:

Please amend claims 1, 3, 10, 16 and 30 as follows:

1. (Amended) A method for detecting or determining delivery and expression of a nucleic acid introduced into a cell [comprising;]comprising:

introducing labelled large nucleic acid molecules that encode a reporter gene into cells;

detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and

measuring the product of the reporter gene as an indication of DNA expression in the cell, whereby delivery and expression of nucleic acid molecules in the cell is detected or determined.

3. (Amended) The method of claim 1, wherein the labelled cells are detected by flow [cytometry]cytometry.

9. [(Twice Amended)] (Thrice Amended) The method of claim 6, wherein [step (a)] the step of introducing labelled large nucleic acid molecules comprises contacting the nucleic acid [molecule] molecules with a delivery agent that comprises a cationic compound.

10. (Twice Amended) The method of claim 9, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [$C_{52}H_{106}N_6O_4.4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$]$C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2 \bullet 4CF_3CO_2H$, $C_{40}H_{84}NO_3P \bullet CF_3CO_2H$, $C_{50}H_{103}N_7O_3 \bullet 4CF_3CO_2H$, $C_{55}H_{116}N_8O_2 \bullet 6CF_3CO_2H$, $C_{49}H_{102}N_6O_3 \bullet 4CF_3CO_2H$, $C_{44}H_{89}N_5O_3 \bullet 2CF_3CO_2H$, $C_{41}H_{78}NO_8P$, $C_{100}H_{206}N_{12}O_4S_2 6 \bullet 8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9 \bullet 13CF_3CO_2H$, $C_{43}H_{88}N_4O_2 \bullet 2CF_3CO_2H$, $C_{43}H_{88}N_4O_3 \bullet 2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

16. (Twice Amended) The method of claim 14, wherein the [cells,]cells are stem cells, nuclear transfer donor cells, tumor cells or transformed cells.

30. (Amended) The method of claim 1, wherein the cell is selected from the group consisting of a primary cell, an immortalized cell, an embryonic cell, a stem cell, a transformed [cells]cell and a tumor cell.

JC97 JUN 16 2003 TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: DE JONG *et al.*

Serial N .: 09/815,981 Group Art Unit: 1636

Filed: March 5, 2001 Examiner: Sullivan, D.M.

For: METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
U.S. Patent and Trademark Office
Alexandria, VA 22313-1450

Sir:

I, Sandra Vanderbyl, declare as follows:

1) I am currently an Associate Scientist at Chromos Molecular Systems, Inc., located at 8085 Lougheed Highway, Burnaby, B.C. V5A IT6 Canada. I have held this position since June 2002 and I have been employed by Chromos Molecular Systems Inc. since April 1998. I earned a Masters of Science (MSc) degree in Biochemistry at the University of Victoria in May 1985.

2) In my position at Chromos Molecular Systems, Inc., (hereinafter Chromos) I have been extensively involved in projects designed to deliver large nucleic acid molecules into mammalian cells and the development of protocols for cell analysis using flow cytometry and microscopy.

3) Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have demonstrated that labeled DNA can be delivered to cells, that the delivery of the labeled DNA can be measured by detection of labeled cells and that the expression of a gene product from the labeled DNA can be measured.

As exemplified by the results shown below, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, one can (i) prepare large labeled nucleic acid encoding a reporter gene; (ii) deliver the labeled nucleic acid to a cell; (iii) measure the delivery of the labeled nucleic acid to the cell; and (iv) determine the expression of the product encoded by the nucleic acid.

U.S.S.N. 09/815,981
DE JONG *et al.*
DECLARATION

I. Materials and Methods

Cell Culture and Chromosome Isolation

The *A*Ces production cell line (CHO E42019), containing a murine-derived artificial chromosome (approximately 120 megabases), was grown in α-minimum essential medium (Life Technologies) with 10% fetal bovine serum (FBS; Can Sera, Rexdale, Ontario) and 0.168 μg/ml hygromycin-B (Calbiochem, La Jolla, CA). The green fluorescent protein (GFP) artificial chromosome was constructed by amplification-dependent *de novo* chromosome formation technique (Csonka et al.,(2000) *J Cell Sci. 113*: 3207-3216) using a Nsi I/Sma I fragment from pIRES-EGFP (Clontech, Palo Alto, CA). An A9 murine cell line containing the GFP artificial chromosome (A9 GFP; approximately 90 Megabases) was maintained in Dulbecco's modified Eagle Medium (DMEM; Life Technologies) plus 10% FBS. Chromosome labeling was accomplished by the addition of Iododeoxyuridine (IdU) or bromodeoxyuridine (BrdU) directly to the culture medium of the CHO E42019 or A9 GFP murine cell lines in the exponential phase of growth. Final concentrations of IdU and BrdU were 0.05-1 μM for continuous labeling of chromosomes (20-24 h) or 10-50 μM for a 15 min pulse labeling of chromosomes. Stock IdU (Sigma, St. Louis, MO) was made in 10mM Tris base buffer (pH 10) and stock BrdU (Sigma) was made in phosphate-buffered saline (PBS). Thirteen to seventeen hours post-plating, exponentially growing CHO E42019 or GFP A9 cultures were blocked in mitosis by a 7h treatment with 1.0 μg/ml colchicine. Chromosomes were then isolated and stained as previously described (DeJong *et al.*, (1999) *Cytometry 35*:129-133), with Hoechst 33258 (2.5 μg/ml and chromomycin A3 (50 μg/ml).

Flow Cytometry for Isolation of *A*Ces

Purification of *A*Ces was performed using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) as previously described (DeJong *et al.*, (1999) *Cytometry 35*:129-133). Chromomycin A3 was excited with the primary laser set at 457 nm, with emission detected using a 475 nm long pass filter. Hoechst 33258 was excited by the secondary UV laser and emission was detected using a 420/44 nm band pass filter. Both lasers had an output of 150 mW. Bivariate distribution showing flow karyotype was accumulated from each sort. *A*Ces were gated from native chromosomes and sorted. Condensing agents (hexylene glycol,

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spermine, and spermidine) were added to the sheath buffer to maintain condensed intact chromosome after sorting.

Labeling Index and Purity of Sorted Chromosomes

IdU or BrdU labeling index of purified *AC*es was determined microscopically. A 10-μl aliquot of approximately 10,000 *AC*es was fixed in 4% formaldehyde solution for 5 min before being air dried onto a clean microscopic slide. The *AC*es were denatured by a 20-30 minute treatment with 2N HCl and then washed two to three times with PBS. Nonspecific binding sites were blocked by submersion in a solution of PBS containing either 4% bovine serum albumin (BSA) or 4% fetal bovine serum (FBS) for a minimum of 10 minutes. A 1:5 dilution (in PBS plus 4% BSA or FBS) of FITC-conjugated anti-IdU/BrdU antibody (Becton Dickinson) in a final volume of 60-100 μl was applied to the slides. The slides were covered with Duraseal plastic strips (Diversified Biotech, Boston, MA) then incubated in a dark humidified chamber at 4°C for 8-24 h. A DNA counterstain of 4',6-diamidino-2-phenylindole dihydrochroride (DAPI; 2 μg/ml in Vectashield (Vector Laboratories, Burlingame, CA) was applied prior to microscopic observation with a Zeiss axioplan 2 microscope equipped for epifluorescence. At least 100 chromosomes were scored to determine the percentage of chromosomes with incorporated IdU or BrdU label. *AC*es isolated without exposure to IdU/BrdU were used as a negative control.

Stability Testing

Sorted *AC*es were stained with propidium iodide (PI;10 μg/ml) and examined by fluorescent microscopy. The *AC*es were assessed for their level of condensation and intactness after the incorporation of IdU or BrdU. Unlabeled *AC*es were used as control.

***AC*es Complex**

One day prior to transfection, Chinese hamster lung fibroblast cells (V79-4) and mouse connective tissue cells [LM(tk-)] were trypsinized and plated into six-well Petri dishes at a per-well density of 250,000 cells and 500,000 cells, respectively. The culture medium was DMEM plus 10% FBS for both cell types. There were approximately 1 million recipient cells per well on the day of transfection. A range of 2-

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40 µl of the LipofectAMINE 2000 (Life Technologies, Burlington, Ontario) transfection agent was added to a standard 1 million *AC*es in 800 µl sheath buffer plus condensing agents followed by a 20 min room temperature incubation. A range of 0.1-18 µl Superfect transfection agent (Qiagen, Inc., Mississauga, Ontario) was added to a standard 1 million *AC*es in 800 µl of sheath buffer plus condensing agents, and incubated 10 min at room temperature.

Transfection

The medium was removed from the wells containing V79-4 or LM(tk-) cells and fresh medium was added: 0.6 ml in wells targeted for Superfect or 2.5 ml for wells targeted for LipofectAMINE 2000-mediated transfer. The LipofectAMINE 2000 (Life Technologies, Burlington, Ontario) or Superfect (Qiagen, Inc., Mississauga, Ontario) *AC*es complexes were then added to the wells for 3 h with Superfect or for 5 h with LipofectAMINE 2000, and the total transfection volume was brought to approximately 5 ml with fresh DMEM plus 10% FBS. The cultures were then incubated at 37°C in a 5% CO_2 incubator. 24-48h post-transfection the cells were washed with PBS and trypsinized. Trypsinization aids in removal of noninternalized chromosome complexes. Cells were fixed in cold 70% ethanol and stored at -20°C prior to antibody labeling.

IdU Antibody Labeling

A standard BrdU staining flow cytometry protocol (Gratzner and Leif (1981) *Cytometry 1*:385-389) was used with some modifications for antibody labeling of IdU labeled cells. Briefly, samples of 1-2 million cells were fixed in 70% cold ethanol. Samples were then centrifuged at 300g for 7-10 min and the supernatant was removed. Cells were then denatured in 1-2 ml of 2N HCl plus 0.5% Triton X-100 for 30 min at room temperature, centrifuged and supernatant removed. This was followed with three to four washes with cold DMEM until the pH indicator in the medium was neutral (pH 7.0), followed by one final wash with cold DMEM plus 5% FBS with centrifugation and supernatant removal between each wash. A blocking/permeabilization buffer consisting of PBS, 0.1% Triton X-100, and 4% FBS was added for 10-15 min before pelleting the sample by centrifugation. Pelleted cells were resuspended in 20 µl of IdU/BrdU FITC-conjugated B44 clone antibody and incubated in the dark for 2 h at room temperature with agitation every 30 min. Cells

were then centrifuged and washed in the blocking/permeabilization buffer and resuspended in 1 ml of PBS. Samples were stored at 4°C.

Flow Cytometry for Detection of Fluorescent IdU

The percentage of transfected cells containing IdU-labeled *AC*es following transfection was determined using a flow cytometer with an argon laser tuned to 488 nm at 400 mW. FITC fluorescence was collected through a standard FITC 530/30-nm band pass filter. Cell populations were gated on the basis of side scatter versus forward scatter to exclude debris and cell doublets. Data were accumulated (20,000-30,000 events) to form bivariate channel distribution showing forward scatter versus log green fluorescence (IdU-FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells such that less than 1% of the negative control cells appeared in the positive region.

IdU Antibody Labeling for Microscopic Analysis

A drop of suspended fixed cell suspension was put on a clean microscope slide and allowed to air dry. Slides were then denatured in 2N HCl plus 0.5% Triton X for 20-30 min at room temperature followed by two to three PBS washes. Nonspecific binding was blocked with a blocking buffer (PBS plus 4% BSA or FBS and 0.1% Triton X) for a minimum of 10 min. Blocking buffer was used to dilute the FITC-conjugated IdU/BrdU antibody 1:5. 60-100 µl of the diluted antibody was applied to each slide. Duraseal plastic strips were overlaid on top of the slides, which were then incubated at 4°C in a humidified, covered box for 8-24 h. DAPI (Sigma; 1 µg/ml in Vectashield) was used as a DNA counterstain. Fluorescence was detected using a Zeiss axioplan 2 microscope equipped for epifluorescence. A minimum of 100 cells was scored.

Enrichment of GFP-Expressing Cell Populations by Fluorescence-Activated Cell Sorting

Cell sorting was carried out in PBS using a FACs vantage flow cytometer. GFP was excited with a 488-nm laser beam and excitation was detected in FL1 using a 500 EFLP filter. Forward and side scattering were adjusted to select for viable cells. Only viable cells were analyzed for GFP fluorescence. Gating parameters were adjusted using wildtype A9 murine cells as negative control.

II. RESULTS

The results presented below demonstrate that, as described in the specification and in Vanderbyl *et al.*, (2001) *Cytometry* 44:100-105, which is of record in the instant application and which is a subsequent publication of the subject matter of this application, labeled large nucleic acid molecules, herein exemplified by IdU-labelled *AC*es, can be delivered to cells and are able to express a reporter gene product, herein exemplified by GFP. Furthermore, the GFP expression obtained with labelled *AC*es is similar to the expression obtained from unlabeled *AC*es.

A) Labelling of *AC*es

The *AC*es containing CHO E42019 cell line was used to compare the incorporation of BrdU versus IdU in the following manner. Exponentially growing monolayer cultures were exposed to either a short 15-min pulse of 10-50 μM IdU or BrdU or alternatively to a 20-24 h exposure of 0.05-1 μM IdU or BrdU. Subsequently, chromosomes were stained with Hoechst 33258 and chromomycin A3 and bivariate karyotype flow profiles were collected for each labeling condition.

Quenching caused by Incorporation of IdU or BrdU caused the Hoescht 33258 stain shifted the entire IdU/BrdU labeled chromosome populations, ~~with BrdU labeled chromosomes exhibiting a greater shift on the bivariate karotype flow profiles~~. Analysis of a range of IdU and BrdU concentrations demonstrated that using lower levels of IdU and BrdU in conjunction with Hoescht 33258 stain provided improved resolution and sorting purity of the *AC*es. ~~A BrdU concentration of 0.05 μM is suitable for isolation of *AC*es~~.

The labeling index of sorted *AC*es was determined by microscopic analysis of ≥ 100 chromosomes. *AC*es given a 15-min pulse label of 50 μM IdU or BrdU had a labeling index of 62%. Sorted *AC*es labeled for 20-24 h with 0.05-1.0 μM IdU or BrdU had labeling indices ranging from 65% to 100%. Twenty-one sorted *AC*es that had been labeled with 0.8 μM IdU in the CHO E42019 cell line gave labeling indices ranging from 93% to 100% with a mean of 97.8 ± 2.62% (Vanderbyl *et al.*, page 102, col. 2 through page 103, col. 1). The absolute values of labeling indices after assessment of IdU or BrdU molarity were not significantly different. The IdU preparations maintained a higher fluorescent intensity. Quality control studies were accomplished using microscopic grading of *AC*es after PI staining. These studies indicate that the samples labeled for 20-24h with IdU were approximately 10-20% less susceptible to decondensation and fragmentation. *AC*es were more stable when labeled with low

concentrations f BrdU or IdU over a 20-24h time period as compared to pulse-labeled samples. These results indicate that 0.4-0.8 μM IdU for 20-24 h is suitable for labeling *ACes* (Vanderbyl *et al.*, page 103, col. 1).

B) Gene Expression from Labeled *ACes*

GFP expression was measured to assess expression from the IdU labeled *ACes*. The *ACes* containing cell line, A9 GFP, was labeled with 24h incorporation of IdU at 0.8 μM and then analyzed for GFP fluorescence by flow cytometry. As a control, GFP expression was measured from unlabeled A9 GFP cells. Two samples were measured from each of the labeled and unlabeled cell populations at 24-96 h postlabeling. The average GFP expression of unlabeled cells was 52.8% while the GFP expression of IdU labeled cells averaged 47.3% (Vanderbyl *et al.*, page 103, col. 1). There was no significant difference between the average GFP expression from a labeled versus unlabeled cell by a Student T-test. The expression data is shown in Table 1 below.

Table 1: Comparison of GFP Expression from Labeled and Unlabeled *Aces*

	Unlabeled	Labeled
Sample 1	39.65%	36.22%
Sample 2	66.01%	58.27%
Average	52.83%	47.25%

C) Chromosome Transfer

To further demonstrate the functionality of the labeled chromosomes, chromosome transfer experiments were performed using either LipofectAMINE 2000 or Superfect reagents. Transfections were performed with a range of dose ratios (reagent:cells). Success of *Aces* transfer was monitored by flow cytometry following antibody labeling with FITC conjugated anti-IdU/BrdU. The FITC-positive fraction of the cell population as analyzed by flow cytometry represented delivery of IdU-labeled *ACes* to recipient cells (see also Figure 2, Vanderbyl *et al.*).

The percent of cells carrying *ACes*, as assessed by both microscopic examination and flow cytometry of the same sample, was determined for and compared

between both LM(tk-) and V79-4 transfections. The percent of *A*Ces-positive cells detected by flow cytometry ranged from 0% to 26% with a mean value of 7.49%. The microscopic analysis of the same samples resulted in an average of 7.68% *A*Ces-positive cells. Linear regression analysis indicated a good correlation ($r^2=0.7$, $n=68$) between microscopically scored and flow cytometry analyzed *A*Ces-positive populations (Vanderbyl *et al.*, page 104, col. 1 and also Figure 4).

III. **Conclusions**

The above experiments demonstrate that large labeled nucleic acids can i) be delivered to cells; ii) delivery can be measured by measuring labeled cells; iii) the labeled nucleic acids express a reporter gene encoded by the nucleic acids; and (iv) the level of reporter gene expression from the labeled nucleic acid is similar to that obtained from unlabeled nucleic acid.

Specifically, the results demonstrate that large DNA encoding a GFP reporter can be labeled with IdU and that the labeled DNA expresses GFP. Furthermore, the GFP expression from labeled DNA is similar to the GFP expression from unlabeled DNA.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

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A Flow Cytometry Technique for Measuring Chromosome-Mediated Gene Transfer

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Background: Using artificial chromosome expression systems (*ACes*), we have developed a unique and rapid screening technique to quantify delivery of foreign DNA into cells in vitro. Delivery was measured within 24 h after transfection, using flow cytometry to detect the transfer of *ACes* labeled with thymidine analogue. This technique can be used to optimize delivery parameters of *ACes* and heterologous DNA into cells and eventually tissue.

Method: Chinese hamster ovary (CHO) cells carrying artificial chromosomes were grown in media supplemented with iododeoxyuridine (IdUrd). The 60-mb artificial chromosome was purified by flow cytometry sorting and transfected into Chinese hamster lung fibroblast cells (V79-4) or mouse connective tissue cells [LM(tk-)] using LipofectAMINE 2000™, a cationic lipid, and Superfect™, a cationic dendrimer. The cells were incubated with an FITC-conjugated anti-bromodeoxyuridine (BrdUrd) antibody and analyzed by flow cytometry. IdUrd-incorporated artificial chromosome expressing green fluorescent protein (GFP) was transfected into V79-4 cells. Delivery was measured at 24 h and GFP⁺ expression was detected at 48 h.

Results: The delivery of intact artificial chromosomes into V79-4 and LMtk- cells was detected within 2 h and up to 48 h post-transfection. Maximum delivery rates of 20% and 14% were observed using LipofectAMINE 2000 and Superfect, respectively. Flow cytometry data correlated with microscopic observations. IdUrd incorporation resulted in less quenching after staining with Hoechst 33258 and chromomycin A3 than BrdUrd incorporation. The fluorescence intensity of the FITC-conjugated anti-BrdUrd antibody was greater with IdUrd-incorporated chromosomes than with BrdUrd-incorporated chromosomes.

Conclusion: The results indicate that IdUrd-labeled artificial chromosomes can be detected 24 h after transfection. This efficient, sensitive, high-throughput detection technique is being used to evaluate and optimize other transfer technologies (e.g., electroporation and sonoporation), different delivery reagents, and protocols in a variety of cells in vitro. This work represents the first step in utilizing artificial chromosomes as nonviral vectors for gene therapy. Cytometry 44:100-105, 2001.

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Key terms: flow cytometry; artificial chromosome; chromosome-mediated gene transfer; bromodeoxyuridine; iododeoxyuridine

INTRODUCTION

Artificial chromosome expression systems (*ACes*) are stable, nonintegrating, expression vectors that can be transferred into a variety of mammalian cells (1). A prototype *ACes* encoding approximately six copies of lacZ and eight copies of hygromycin-B resistance genes has been characterized. The *ACes*, due to a high AT/GC ratio, are easily identified and purified using chromomycin A3 and Hoechst 33258 stains and a dual laser beam flow cytometer (2). Transfection of isolated metaphase chromosomes into target cells is possible but transfer efficiencies fluctuate depending on the recipient cell, chromosome characteristics, and transfection technique used (3). To facilitate the use of *ACes* for gene transfer, high transfection efficiencies are required. A method for screening and evaluating the early stages of the transfection process would be desirable in the development of alternative delivery techniques.

Cellular chromosome uptake is a necessary first step in the transfection process and the method we describe here can be used to identify the optimal conditions for this to occur. Microscopic techniques for visualizing DNA transfer using bromodeoxyuridine (BrdUrd; 4,5) are time consuming, restricted by the large sample size required to detect low levels of transfer, and limited by the necessity for manual scoring. A flow cytometry technique would permit determination of low transfection rates, as large numbers of cells are easily analyzed, and would offer the speed of data collection desirable for a screening protocol. Current flow cytometry protocols require the transfer vector to contain specific reporter genes that are either

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fluorescent themselves or can act on a substrate to produce a fluorescent byproduct. They must be optimally expressed in order for transfer to be identified (6-8). Studies using specific fluorescent DNA dyes can only measure kinetics of DNA uptake during the first hours of transfection (9). We describe an efficient flow cytometry technique where iododeoxyuridine (IdUrd)-labeled *ACes* are detected after transfer into recipient cells. The procedure requires only that the transfer vector is able to integrate thymidine analogues. The transferred IdUrd-labeled *ACes* are immunologically tagged using FITC-conjugated anti-BrdUrd/IdUrd antibody and quantified by flow cytometry. Although standard transfection colony analysis can take from 4 to 6 weeks, transfer of IdUrd-labeled *ACes* can be evaluated within 2-3 days post-transfection. Incorporation of low levels of analogue does not appear to affect expression of *ACes* reporter genes or the stability of the chromosome.

Two transfection agents, LipofectAMINE 2000 (Life Technologies, Burlington, Ontario, Canada) and Superfect (Qiagen, Valencia, CA), were used to transfect IdUrd-incorporated *ACes* into cell lines. LipofectAMINE 2000 reagent is a proprietary liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Superfect reagent consists of activated dendrimer molecules with a defined spherical architecture.

MATERIALS AND METHODS

Cell Culture and Chromosome Isolation

The *ACes* production cell line (CHO E42019) was grown in α-minimum essential medium (Life Technologies) with 10% fetal bovine serum (FBS; Can Sera, Rexdale, Ontario) and 0.168 μg/ ml hygromycin-B (Calbiochem, La Jolla, CA). The green fluorescent protein (GFP) artifical chromosome was constructed by deletion of a Nsi 1/Sma I fragment from pIRES-EGFP (Clontech, Palo Alto, CA) using the amplification-dependent de novo chromosome formation technique (10). The GFP artificial chromosome in an A9 murine cell line was maintained in Dulbecco's modified Eagle Medium (DMEM; Life Technologies) plus 10% FBS. IdUrd or BrdUrd was added directly to the culture medium of the CHO E42019 or A9 GFP murine cell lines in the exponential phase of growth. Stock IdUrd (Sigma, St. Louis, MO) was made in tris base buffer (pH 10) and stock BrdUrd (Sigma) was made in phosphate-buffered saline (PBS). Final concentrations were 0.05-1 μM for continuous label of chromosomes (20-24 h) or 10-50 μM for 15 min pulse label of chromosomes. After 13-17 h, exponentially growing CHO E42019 or GFP A9 cells were blocked in mitosis with 1.0 μg/ml colchicine for 7 h. Chromosomes were then isolated and stained as previously described (2); with Hoechst 33258 (2.5 μg/ml) and chromomycin A3 (50 μg/ml).

Flow Cytometry for Isolation of *ACes*

Purification of *ACes* was performed using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Chromomycin A3 was excited with the primary laser set at 457 nm, with emission detected using a 475-nm long pass filter. Hoechst 33258 was excited by the secondary UV laser and emission was detected using a 420/44-nm band pass filter. Both lasers had an output of 150 mW. Bivariate distribution showing flow karyotype was accumulated from each sort. *ACes* were gated from other chromosomes and sorted. Condensing agents (hexylene glycol, spermine, and spermidine) were added to the sheath buffer to maintain condensed intact chromosome after sorting.

Labeling Index and Purity of Sorted Chromosomes

IdUrd or BrdUrd labeling index of purified *ACes* was determined microscopically. A 10-μl aliquot of approximately 10,000 *ACes* was fixed in 4% formaldehyde solution for 5 min before being air dried on a clean microscopic slide. Air-dried slides were denatured in coplin jars with 2N HCl for 20-30 min at room temperature and then washed two to three times with PBS. Nonspecific binding was blocked with PBS plus 4% bovine serum albumin (BSA) or serum for a minimum of 10 min. A 1:5 dilution (in PBS plus 4% BSA or serum) of FITC-conjugated IdUrd/BrdUrd antibody (Becton Dickinson) with a final volume of 60-100 μl was applied to the slides. Duraseal plastic strips (Diversified Biotech, Boston, MA) were overlaid on top of the slides, which were kept in the dark at 4°C in a humidified covered box for 8-24 h. 4', 6-diamidino-2-phenylindole dihydrochroride (DAPI) stain at a concentration of 2 μg/ml in Vectashield (Vector Laboratories, Burlingame, CA) was used as a DNA counterstain. Fluorescence was detected using a Zeiss axioplan 2 microscope equipped for epifluorescence. At least 100 chromosomes were scored to determine the percentage of incorporated IdUrd or BrdUrd label. Unlabeled *ACes* were used as negative control.

Stability Testing

A drop of sorted *ACes* was diluted in 0.5 μl of 10 μg/ml propidium iodide (PI) onto a microscope slide. Chromosomes were analyzed under a fluorescent microscope for their ability to remain intact and condensed after the incorporation of IdUrd or BrdUrd. Unlabeled *ACes* were used as control.

ACes Complex

The day before the transfection, Chinese hamster lung fibroblast cells (V79-4) were trypsinized and plated at a density of 250,000 cells per well in a six-well Petri dish in DMEM plus 10% FBS. Experiments using mouse connective tissue cells [LM(tk-)] were seeded at a density of 500,000 cells per well. There were about 1 million recipient cells per well on the day of transfection. When using LipofectAMINE 2000, 2-40 μl of the transfection agent

was added to 1 million *AC*es in 800 μl sheath buffer containing condensing agents, for 20 min at room temperature. Prior to Superfect transfection, 0.1–18 μl of Superfect was added to 1 million *AC*es in 800 μl of sheath buffer containing condensing agents for 10 min at room temperature.

Transfection

The medium was removed from the wells containing V79-4 or LM(tk-) cells and fresh medium was added: 0.6 ml in wells targeted for Superfect or 2.5 ml for wells targeted for LipofectAMINE 2000-mediated transfer. The LipofectAMINE 2000 or Superfect *AC*es complexes were then added to the wells drop-wise and gently mixed by swirling the plate. After 3 h with Superfect and 5 h with LipofectAMINE 2000, the total transfection volume was brought to approximately 5 ml with fresh DMEM plus 10% FBS. After 24–48 h at 37°C in a 5% CO_2 incubator, the cells were washed with PBS and trypsinized. Trypsinization aids in removal of noninternalized chromosome complexes (9,11). Cells were fixed in cold 70% ethanol and stored at -20°C prior to antibody labeling.

IdUrd Antibody Labeling

A standard BrdUrd staining flow cytometry protocol (12) was used with some modifications. Briefly, between each step, samples were centrifuged at 300g for 7–10 min and the supernatant was removed. Samples of 1–2 million cells were fixed in 70% cold ethanol. Cells were then denatured in 1–2 ml of 2N HCl plus 0.5% Triton X-100 for 30 min at room temperature. This was followed with three to four washes with cold DMEM, until the pH indictor in the medium was neutral (pH 7.0), and then one final wash with cold DMEM plus 5% FBS. A blocking/permeabilization buffer consisting of PBS, 0.1% Triton X-100, and 4% FBS was added for 10–15 min before pelleting the sample by centrifugation. Pelleted cells were resuspended in 20 μl of IdUrd/BrdUrd FITC-conjugated B44 clone antibody and incubated in the dark for 2 h at room temperature. Cells were agitated every 30 min. Cells were then centrifuged and washed in the blocking/permeabilization buffer and resuspended in 1 ml of PBS. Samples were stored at 4°C.

Flow Cytometry for Detection of Fluorescent IdUrd

The percentage of transfected cells containing IdUrd-labeled *AC*es was determined using a flow cytometer with an argon laser tuned to 488 nm at 400 mW. FITC fluorescence was collected through a standard FITC 530/30-nm band pass filter. Cell populations were gated on the basis of side scatter versus forward scatter to exclude debris and doublets. Data were accumulated (20,000–30,000 events) to form bivariate channel distribution showing forward scatter versus log green fluorescence (IdUrd -FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells such that less than 1% appeared in the positive region.

IdUrd Antibody Labeling for Microscopic Analysis

A drop of fixed cell suspension was put on a clean microscope slide and allowed to air dry. Slides were then denatured in coplin jars with 2N HCl plus 0.5% Triton X for 20–30 min at room temperature and washed two to three times with PBS. Nonspecific binding was blocked with a blocking buffer (PBS plus 4% BSA or serum and 0.1% Triton X) for a minimum of 10 min. A 1:5 dilution of FITC-conjugated IdUrd/BrdUrd antibody diluted in blocking buffer with a final volume of 60–100 μl was applied to the slides. Duraseal plastic strips were overlaid on top of the slides, which were kept in the dark at 4°C in a humidified covered box for 8–24 h. DAPI (Sigma) at a concentration of 1 μg/ml in Vectashield was used as a DNA counterstain. Fluorescence was detected using a Zeiss axioplan 2 microscope equipped for epifluorescence. A minimum of 100 cells was scored.

Enrichment of GFP-Expressing Cell Populations by Fluorescence-Activated Cell Sorting

Cell sorting was carried out in PBS using a FACs vantage flow cytometer. GFP was excited with a 488-nm laser beam and excitation was detected in FL1 using a 500 EFLP filter. Forward and side scattering were adjusted to select for viable cells. Only viable cells were analyzed for GFP fluorescence. Gating parameters were adjusted using wild-type A9 murine cells as negative control.

RESULTS

Optimal Chromosome Stability and IdUrd/BrdUrd Labeling Index

A comparison was made between the incorporation of BrdUrd and IdUrd into the *AC*es containing CHO E42019 cell line. Exponentially growing monolayer cultures were exposed to a short 15-min pulse of 10–50 μM IdUrd or BrdUrd and a continuous exposure of 20–24 h with 0.05–1 μM IdUrd or BrdUrd. Chromosomes were stained with Hoechst 33258 and chromomycin A3 and a bivariate karyotype flow profile was collected. Figure 1 portrays the typical flow karyotypes of the chromosomes labeled by continuous exposure with 0.8 μM IdUrd or BrdUrd for the *AC*es containing CHO E42019 cell line. The BrdUrd chromosome karyotype (Fig. 1a) showed Hoechst 33258 quenching caused by insertion of the thymidine analogues (13), with a lesser amount of quenching using equimolar quantities of IdUrd (Fig. 1b). Because the quenching shifted the chromosome population as a whole, the labeled *AC*es could be sorted as effectively as unlabeled preparations. Improvements in the BrdUrd flow karyotype could be achieved by lowering the concentration of BrdUrd continuous label to 0.05 μM (data not shown). However, the labeling index of the sorted *AC*es preparation was decreased to 68%.

The labeling index of sorted *AC*es was determined microscopically with a minimum of 100 chromosomes scored. A labeling index of 62% was determined for *AC*es given a 15-min pulse label with 50 μM IdUrd or BrdUrd. Sorted *AC*es, continuously labeled for 20–24 h with 0.05–

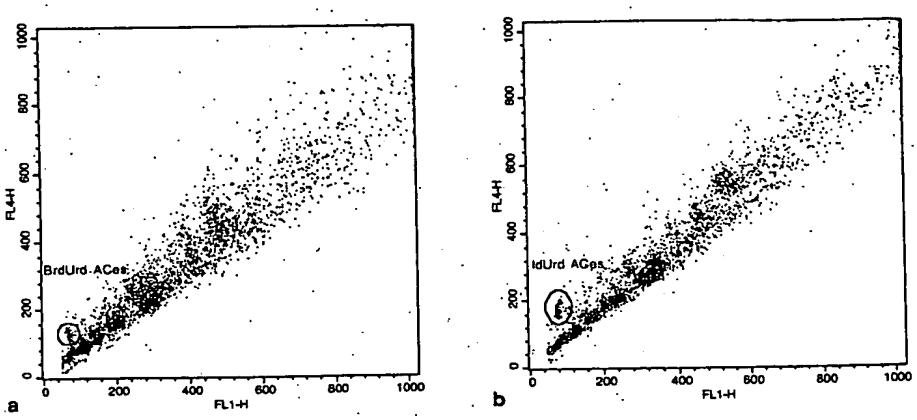


FIG. 1. Comparison bivariate flow karyotypes (chromomycin A3, FL1-H, versus Hoechst 33258, FL4-H) of a: BrdUrd and b: IdUrd 0.8 μM continuously labeled artificial chromosome CHO E42019 production cell line. Circled areas represent gate used for sorting artificial chromosomes.

1.0 μM IdUrd or BrdUrd, had labeling indexes ranging from 65% to 100%. *ACes* sorted from 21 samples labeled with 0.8 μM IdUrd in the CHO E42019 cell line for 20–24 h had labeling indexes ranging from 93% to 100% with a mean of 97.8 ± 2.62%. Equimolar IdUrd or BrdUrd labeling index absolute values were not significantly different. However, the IdUrd preparations had a higher fluorescent intensity. Quality control studies, using microscope grading of the stability of the chromosomes by appearance with PI staining, indicated that the continuous IdUrd-labeled samples were approximately 10–20% less susceptible to unraveling and fragmentation. *ACes* were more stable at low-level continuous incorporation for either BrdUrd or IdUrd when compared with pulse-labeled samples. Our results indicate that the optimal labeling condition for *ACes* is 0.4–0.8 μM IdUrd for 20–24 h. Continuous IdUrd incorporation at 0.8 μM for 24 h did not affect GFP expression in the A9 murine cell line. GFP expression of unlabeled A9 murine cells was an average of 52.8% compared with GFP expression of IdUrd continuously labeled cells being 47.3% at 24–96 h postlabeling.

Chromosome transfer was performed using LipofectAMINE 2000 or Superfect, starting at dose ratios known to give stable transformants with a minimum transfer efficiency of 10^{-6} and then broadening the range. The percent positive FITC population using flow cytometry represented delivery of IdUrd-labeled *ACes* to recipient cells. The dose-response curves showing maximum delivery values of the *ACes* into V79-4 cells at 24 h posttransfection are shown in Figure 2. LipofectAMINE 2000 (Fig. 2a) caused a sigmoidal dose curve with a plateau at 20% positive cells in the range of 30–40 μl of the transfection agent. Superfect (Fig. 2b) shows a dose-response curve with a 14% delivery peak between 4 and 6 μl of the transfection agent.

Figure 3 is a representative histogram of V79-4 Chinese hamster cells using Superfect as the transfection agent, which shows green FITC fluorescence that correlates to delivery of IdUrd *ACes*. As indicated by the typical flow profile, there is at least a log magnitude difference between the fluorescent intensity of the negative or control population (A) and the positive population (B).

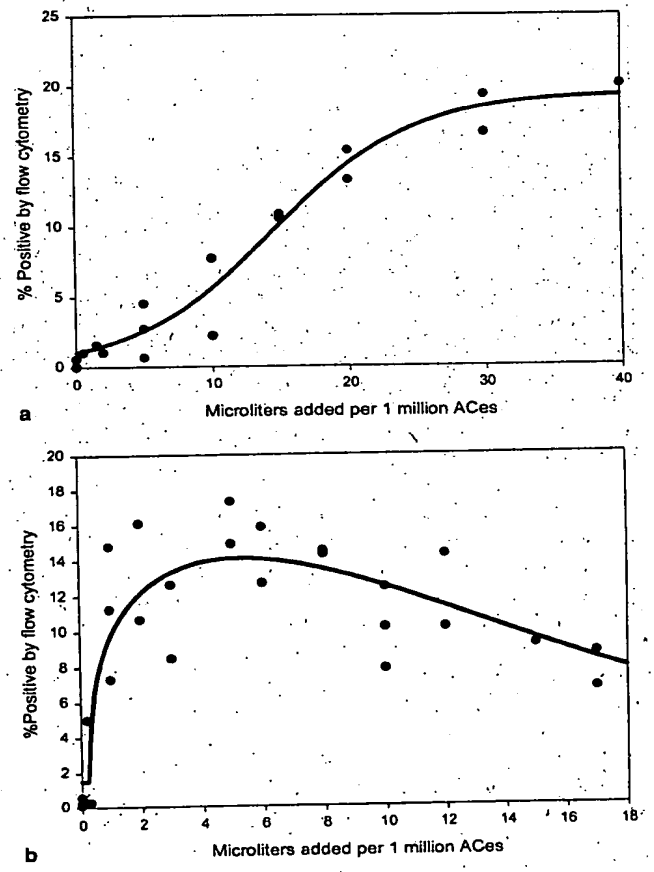


FIG. 2. The dose-response curves showing maximum delivery values of the IdUrd-labeled *ACes* into V79-4 cells at 24 h posttransfection. Positive population is determined by flow cytometric analysis of the green fluorescent signal from BrdUrd/IdUrd FITC-conjugated antibody. Doublets and debris have been gated from analysis. a: LipofectAMINE 2000 data are fitted with a sigmoidal curve ($r^2 = 0.94$) with a plateau at 20% delivery of the artificial chromosome. b: Superfect shows a dose-response curve with a peak at 14% delivery ($r^2 = 0.8$).

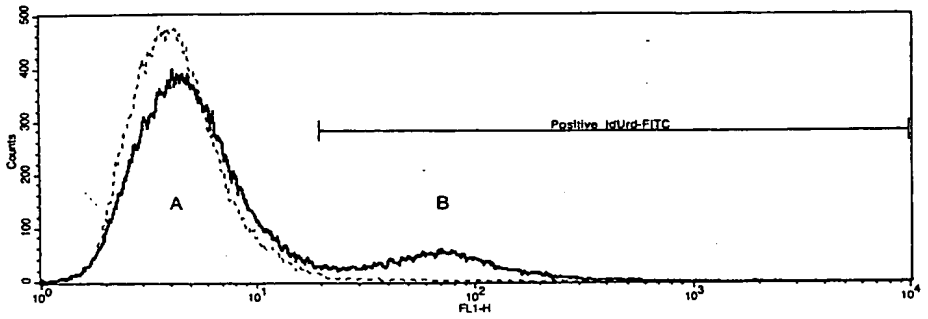


FIG. 3. Representative flow histogram of V79-4 Chinese hamster cells showing log green FITC fluorescence (FL1-H) correlating to IdUrd *A*Ces delivery using Superfect as the transfection agent. Solid line represents the transfected cells and shows that 14.9% of the cells express a log magnitude increase in fluorescence compared with the controls cells (dotted line).

There was only a minimal decrease in fluorescent intensity comparing samples collected at 4–48 h post-transfection (data not shown). Control cell populations of transfections with unlabeled *A*Ces or IdUrd labeled *A*Ces added without transfection agent ranged from 0.2% to 1% positive cells due to nonspecific antibody binding.

To verify that the positive population was representative of the transfer of at least one *A*Ces per cell, the positive populations were sorted and then analyzed microscopically. At 24 h posttransfection, the fluorescent signals were mostly nuclear or perinuclear, mainly one *A*Ces per cell, and occasionally (<5%) two *A*Ces per cell or smaller signals were detected.

A data set was collected from LM(tk-) and V79-4 transfections comparing independently evaluated positive values (percent of *A*Ces carrying cells) determined microscopically and by flow cytometry from the same sample (Fig. 4). The percent of positive cells detected by flow cytometry ranged from 0% to 26%. Linear regression analysis indicates a good correlation ($r^2 = 0.7$, $n = 68$) between microscopically scored and flow cytometry-analyzed positive populations. The mean data set for flow cytometry-derived positive values was 7.49% compared with mean for microscopic samples of 7.68%.

DISCUSSION

Thymidine analogue integration into replicating DNA is a widely known and used technique. The applications are diverse: cell cycle studies, with thymidine analogue incorporation during S phase allowing for the tracking of proliferating cells; or at the chromosome level, looking at DNA exchange by comparing labeling of sister chromatids. However, use of thymidine analogues to track foreign DNA has been limited to microscopic analysis of samples transfected with DNA (3,4) or to the transfer of sperm DNA into embryos (14). Presented here is a unique flow cytometry application to track foreign DNA by following the transfer of IdUrd-labeled *A*Ces into murine or Chinese hamster cell lines.

Our *A*Ces contain large amounts of AT-rich mouse major satellite DNA, interrupted by bands of reporter genes. The *A*Ces used in this study is about 60 megabases in size and contains the lacZ and hygromycin resistance genes (15). Because satellite DNA is proportionally AT rich, the artificial chromosome is a good platform to investigate the possibility of tracking thymidine analogue-labeled chromosomes using flow cytometry.

BrdUrd is the more commonly used and recognized thymidine analogue. However, IdUrd was found to be superior to BrdUrd for our application. IdUrd has a higher affinity for the commercially available B44 clone FITC-conjugated antibody (16) and it less readily salvaged for DNA synthesis from dead cells (17,18). The IdUrd-induced DNA strand breaks caused by exposure to UV light in the presence of Hoechst 33258 are a magnitude lower than BrdUrd (19). As shown in Figure 1, both BrdUrd and IdUrd- labeled *A*Ces can be sorted from the CHO E42019 production cell line. However, BrdUrd showed more Hoechst 33258 quenching, which can affect *A*Ces sort purity. Both BrdUrd and IdUrd integrate with a high labeling index. However, IdUrd shows a higher fluorescence antibody staining intensity (16). Microscopically, IdUrd

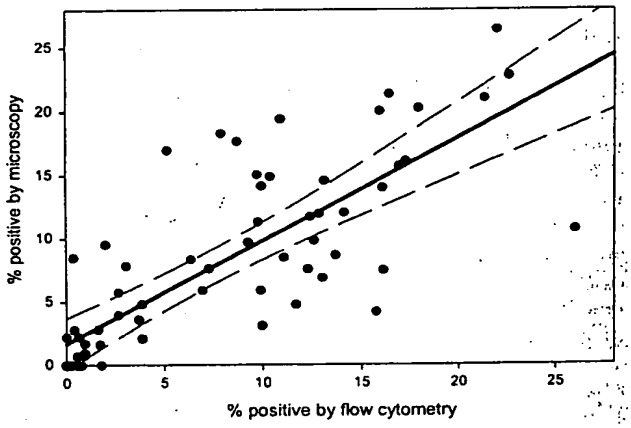


FIG. 4. Correlation between microscopic and flow analysis of a positive transfected population determined by a fluorescent signal from IdUrd-labeled *A*Ces detected by FITC-conjugated antibody. A data set ($n = 68$) from both LM(tk-) and V79-4 transfections using Superfect or LipofectAMINE 2000 was collated. Microscopic and flow analyses were done from the same sample but were independently analyzed. A plot of linear regression is indicated by a bold line and 99% confidence limits are indicated by dashed lines. Linear regression analysis shows good correlation ($r^2 = 0.7$) between microscopically scored and flow cytometry-analyzed positive populations. The data set for flow cytometry-derived positive values had a mean of 7.49% with a median value of 6.68% compared with the mean for microscopic samples of 7.68% with a median of 6.37%.

continuously labeled chromosomes (20-24 h) were more stable and condensed than the BrdUrd or 15-min pulse-labeled counterparts. For the above reasons, 0.4-0.8 μM of IdUrd incorporation for 20-24 h was determined to be our standard labeling conditions to follow *A*Ces transfection.

The use of flow cytometry as a tool to detect the presence of one or more transfected chromosomes in a recipient cell is shown in our study. Microscopic analysis of the sorted positive green fluorescence population confirms that we are measuring the transfer of a single chromosome with a high percentage of purity. There is good correlation between microscopic scoring and flow cytometry green FITC-positive populations. This protocol can be used as a rapid screening tool to test the potential of delivery methods and to optimize dose ranges of transfection agents for foreign DNA, including *A*Ces. The protocol is simple and the data can be analyzed within 24 and 48 h after transfection, which makes it appealing over traditional time-consuming colony-forming transfection studies. The measurement of chromosome transfer is direct and is not dependent on the expression of a reporter gene such as a selectable marker or fluorescent protein. The IdUrd delivery protocol has advantages over GFP expression because it can measure absolute values of chromosome transfer and is not hindered by cell autofluorescence, maintaining optimal expression conditions and the problems of differentiating wild-type cells from low-expressing populations (7). The IdUrd protocol requires fixed cells and is not appropriate for studies in which sample viability must be maintained postdetection.

Flow analysis offers the potential of collecting data with superior accuracy due to the ease at which large numbers of events are collected. The positive values are instrument derived and are less susceptible to judgement errors. Microscopic analysis is limited by the time involved for scoring positive events and sample size is restrictive. For this study, 100-300 cells were scored microscopically, which took about 10-15 min per slide, compared with 20,000-30,000 events collected by flow cytometry within seconds. However, because the flow cytometer integrates the fluorescent signal over the entire cell, fragmented, single, or multiple transfected chromosomes could not be easily differentiated. Microscopic analysis gave an indication of signal size and cellular location and, in some cases, identified a portion of the positive cell population (<5%) that contains fragmented (small FITC signal) or multiple *A*Ces.

We have presented data detailing the use of flow cytometry to detect the presence of an *A*Ces in a transfected cell. The protocol can be used as a rapid screening tool to optimize chromosome transfer conditions. Because the method only requires that the transfer vector be able to integrate thymidine analogues, it suggests the use of this method for other platforms such as cDNA and plasmids (4) or as an evaluation step to enable the investigation of low transfection rates. The fact that the analogue integration did not adversely affect GFP protein expression suggests its possible use as a tool for mapping the biological events between the initial stages of delivery and early gene expression. This technique could be used to determine the transfection efficiency using a variety of transfection methods, such as electroporation and sonoporation, and to compare transfection products in a variety of cell lines.

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